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Characterisation and activation of Foxp3+ and Tr1 regulatory T cells in nasopharynx-associated lymphoid tissue by *S. pneumoniae*

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Abstract

Streptococcus pneumoniae (pneumococcus) is associated with high morbidity and mortality, particularly in young children under the age of 5 and elderly people. Currently, vaccinations against S. pneumoniae are polysaccharide based. While these are effective, they are limited in several ways including their serotype coverage. Recently, research focus has moved to looking at the development of a vaccine that could be effective across all *S. pneumoniae* serotypes and as such pneumococcal proteins which are conserved across all serotypes are being investigated for possible inclusion in a vaccine.

Regulatory T cells are known to have an important role in the modulation of immune responses to pathogens. Previous studies have shown that pneumococcal proteins may be involved in the activation of Foxp3+ Treg cells in tonsillar tissue. Pneumococcal whole cell antigen (WCA) has been shown to promote an increased number of Foxp3+ Treg cells in tonsillar tissue which suggests there is a pneumococcal component which influences numbers of this cell type.

Type 1 regulatory T (Tr1) cells are an inducible subset of regulatory T cells that play a role in promoting and maintaining tolerance. The secretion of high levels of IL-10 is the main mechanism by which Tr1 cells exert their effects. In 2013, Roncarolo et al showed that the co-expression of cell surface markers CD49b and LAG-3 could be used to identify Tr1 cells and for the purposes of this study it was the co-expression of these cell surface markers that was used to identify the numbers of Tr1 cells present[1].

The frequency of Foxp3+ Treg and Tr1 cells in tonsillar tissue and peripheral blood samples from both adults and children were analysed for the intracellular expression of Foxp3 and IL10 respectively. Tr1 cells were also characterised by the co expression of cell surface markers CD49b and LAG3. Tonsillar MNC and PBMC were also stimulated using pneumococcal concentrated culture supernatant (CCS), a toxoid of pneumolysin (W433F) and purified polysaccharide capsule (T3P and 6B).

Numbers of Foxp3+ Treg and Tr1 cells were shown to be significantly higher in tonsillar tissue than in peripheral blood in both adult and children samples. After stimulation of tonsillar MNC and PBMC, , using WT pneumococcal CCS a significant increase in numbers of Foxp3+ Treg and Tr1 cells was shown compared to a negative control. The use of isogenic mutant strains deficient in pneumolysin (Ply-/-) and capsule (Cap-/-) showed a significantly lower activation of Foxp3+ Treg and Tr1 cells. This suggests that pneumolysin and capsule may be important for the activation of Foxp3+ Treg and Tr1 cells. The same experiments were carried out using a toxoid of pneumolysin (W433F) and purified capsular polysaccharide (T3P and 6B) and the results showed that both W433F and T3P and 6B were able to activate significantly more Foxp3+ Treg and Tr1 cells.

TGF-β has been shown to be important for the induction of Foxp3+ Treg cells. TGF-β is released in an inactive form and must be activated to exert its suppressive effects. The integrin αvβ8 has been shown to have a role in the activation of TGF-β. Tonsillar MNC were stimulated by pneumococcal CCS and the supernatant was collected and analysed using ELISA. Cells were pre-incubated using anti-TGF-β and anti-αvβ8. Intracellular cytokine staining of samples was also performed which determined that TGF-β concentration did correlate with the presence or absence of Foxp3+ Treg cells and that avb8 did appear to play a role in the activation of TGF-β

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Declaration

The original work of the author is presented in this thesis unless stated otherwise. All laboratory experiments described here have been performed by the author in The Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

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List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
BA	Blood agar
BALT	Bronchial-associated lymphoid tissue
BFA	Brefeldin A
BSA	Bovine serum albumin
CBD	Choline binding domain
CBP	Choline binding protein
CbpA	Choline binding protein A
CCS	Concentrated culture supernatants
cfu	Colony forming units
CO2	Carbon dioxide
CPCV23	23-valent capsular polysaccharide vaccine
CPS	Capsular polysaccharide
CRP	C-reactive protein
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocytes associated antigen-4
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead-box P3
FSC	Forward scatter
g	Centrifugal force
g	Gram
GALT	gastrointestinal-associated lymphoid tissue
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H2O2	Hydrogen peroxide
H2SO4	Sulphuric acid
hr	Hour
HRP	Horse radish peroxidase
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
iTreg	Inducible Treg cells
LAG-3	Lymphocyte activation gene-3
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LytA	Autolysin A
M	Molar
mA	Milliampere
MALT	Mucosal-associated lymphoid tissue
MAC	Membrane attack complex
Mg	Milligram
min	Minute
ml	Millilitre

MNC	Mononuclear cell
NALT	Nasopharynx-associated lymphoid tissue
NaOH	Sodium hydroxide
ng	Nanogram
NK cell	Natural killer cell
NLR	Nod-like receptor
NP	Nasopharyngeal
nTreg	Natural Treg cell
OD	Optical density
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine
PdB	Genetically detoxified pneumolysin
PE	Phycoerythrin
pH	Power of hydrogen
Ply	Pneumolysin
PMN	Polymorphonuclear leucocytes
PRR	Pathogen recognition receptor
PsaA	Pneumococcal surface adhesion A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
rPly	Recombinant pneumolysin
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SSC	Side scatter
STGG	Skim milk, tryptone, glucose, glycerol transport medium
TCR	T-cell receptor
TGF-β	Transforming growth factor
Th	T helper
Th1	T helper 1 cell
Th17	T helper 17 cell
Th2	T helper 2 cell
Th3	TGF-β-secreting T helper 3 cell
THB	Todd-Hewitt-broth
TLR	Toll like receptor
Tr1	IL-10- secreting T regulatory 1 cell
Treg	Regulatory T cell
TSB	Tryptone soya broth
U	Units
URT	Upper respiratory tract
V	Volts
WCA	Whole cell antigen
WHO	World Health Organization
wt	wild type
yr	year
µg	Microgram
µl	Microlitre

Chapter one

General Introduction

1.1 Natural immunity to *Streptococcus pneumoniae*

Streptococcus pneumonia (pneumococcus) is an important human pathogen that is responsible for several invasive and non-invasive diseases which range in severity from mild otitis media to severe meningitis and pneumonia[2]. Pneumococcal disease is particularly prevalent in high risk groups including the elderly and young children and is a leading cause of childhood morbidity and mortality worldwide. Pneumococcal infection is particularly a problem in developing countries where infection rates are higher than in developed countries.

Pneumococcal transmission is through direct contact with respiratory droplets containing the bacteria. This can be via infected patients or healthy individuals who carry the bacteria. Not everyone who is a carrier will present with symptoms of infection. The bacteria can persist without being cleared and become part of the normal microbiota of the individual with 5-40% of individuals carrying the bacteria as part of their normal flora[3]. Transient carriage is generally harmless in healthy individuals, but the longer carriage persists, the higher the chance of the pneumococcus being able to disseminate from the nasopharynx into the normally sterile respiratory tract and cause infection[4].

Colonisation by pneumococcus is a prerequisite for invasive disease, acting as the first point of contact between the bacteria and the host. From here the pneumococcus has the potential to be able to reach the lungs, blood or cross the blood brain barrier and infect the brain. The primary site of pneumococcal colonisation is the nasopharynx. Dissemination of bacteria from the mucosal site is the cause of further severe infection such as septicaemia and meningitis. The colonised nasopharynx also acts as a reservoir of the bacteria allowing spread through the community.

It is thought that almost all children are colonised by at least one pneumococcal serotype in their first couple of years of life[5]. The incidence of pneumococcal colonisation has been

shown to decrease with age, but it is not uncommon for children to be repeatedly colonised with numerous serotypes[6, 7]. This colonisation has potential immunogenic effects, priming both mucosal and systemic immune responses which confer protection from further colonisation events and invasive disease. Colonisation at a young age provides immunological memory against pneumococcal antigens which may provide protective effects against colonisation in later life and may thus contribute to the decrease in colonisation events seen with age[8].

Vaccination is the most effective way of protecting against pneumococcal disease, but the problem has been the large number of serotypes that have to be protected against. There are more than 90 known serotypes and current vaccines are only effective against a small proportion of these[9]. There are 2 types of pneumococcal vaccines currently available; polysaccharide vaccines and protein conjugate vaccines both of which have narrow serotype coverage. Pneumococcal polysaccharide capsule has long been known to induce protective immunity. The first polysaccharide-based vaccine was commercially available after the end of the First World War and targeted four serotypes[10]. Later, a 23-valent polysaccharide vaccine was licensed[11]. Polysaccharide vaccines are limited in that they produce a T cell independent immune response and are therefore ineffective in children under 2 years of age who are the most vulnerable to pneumococcal colonisation[4, 12]. Due to this, protein conjugate vaccines have been developed which contain polysaccharide conjugated to a protein that induces a T-cell dependent response, and immunological memory. Protein conjugate vaccines are effective, but they are expensive and have narrow serotype coverage. The use of vaccines that target specific serotypes have led to a decrease in serotypes contained in the vaccine but has seen the emergence of different serotypes not contained in the vaccine[13]. Efforts are now being made to overcome the limitations faced by current pneumococcal vaccines by developing a protein-based vaccine which would target most, if not all serotypes[14, 15]. In addition to targeting many more serotypes, a

protein-based vaccine is thought to be commercially easy to produce and a lot cheaper than current vaccines[12]. As such current research is focusing on the identification of suitable proteins for incorporation into a pneumococcal protein vaccine.

1.2 Microbiology of *Streptococcus pneumoniae*

S. pneumoniae are gram positive encapsulated cocci. They are most commonly seen in pairs referred to as diplococci but can also occur as short chains or as solitary cocci[3, 16]. They are non-motile facultative anaerobes with the size of individual bacterium ranges from 0.5-1.25 um. *S. pneumoniae* are catalase negative, a characteristic shared with other streptococci, but can be distinguished from other group A streptococci by their α-haemolytic colonies on blood agar. Haemolysis is the breakdown of red blood cells and alpha haemolysis is characterised by the area of agar underneath the colony turning green[17].

On blood agar plates *S. pneumoniae* produce glistening, raised colonies which are approximately 1mm in diameter. Over time the colonies tend to flatten and depress due to the presence of an autolytic enzyme. *S. pneumoniae* are commonly differentiated from other α-haemolytic bacteria using their colony morphology as well as using several biochemical tests including sensitivity to optochin. *S. pneumoniae* is susceptible to the chemical optochin and will not grow in its presence[18]. To test optochin susceptibility, a disc which has been impregnated with optochin is placed on a plate which is seeded with the microbe being investigated. Upon incubation of the plate the bacteria will multiply to form visible colonies but when the microbe is sensitive to optochin there will be a halo formed around the disc where the bacteria has been unable to grow.

Almost all pathogenic strains of *S. pneumoniae* have a polysaccharide capsule which plays a key role in its virulence and is the basis for serotype differentiation[19]. Each capsular polysaccharide reacts with its specific antisera causing the 'Quelling' or swelling reaction.

This describes the microscopic appearance of the pneumococcal capsule after the polysaccharide has combined with its specific antisera. Because of the reaction the capsule appears to swell hence the name. To date more than 90 different capsular serotypes of *S. pneumoniae* have been identified. 90 were originally identified [20] and a further (2/3) more recentlY[21].

Like other gram-positive bacteria, *S. pneumoniae* have a cell wall composed peptidoglycan, techoic acid residues and lipotechoic acid residues. The peptidoglycan layer is a thick rigid structure composed of several peptidoglycan layers combined[22]. This is one of the defining characteristics of a gram-positive bacteria as gram negative bacteria have a thin peptidoglycan layer[22]. Both techoic and lipotechoic acids are negatively charged which give them a role in binding and regulating the movement of positive ions into and out of the cell[23]. Lipotechoic acid residues are directly bound to the plasma membrane and traverse the peptidoglycan layer while techoic acid residues do not touch the plasma membrane[23].

1.3 Pathogenesis of *Streptococcus pneumoniae*

Pneumococci can spread internally and externally from their site of colonisation the nasopharynx. Internal spread of bacteria leads to either invasive or non-invasive infection of the host, whilst external spread of bacteria via respiratory droplets leads to infection of a new host. Spread of bacteria within the host can occur by several different pathways. Bacteria can spread through into the respiratory tract, middle ear cavity, central nervous system (CNS) or blood stream and cause inflammatory responses in each of these locations leading to either mucosal infection or invasive disease.

1.3.1 Otitis media: Otitis media is a middle ear infection that causes inflammation, redness and build of fluid behind the ear drum. Any age group can be affected by otitis media but very young children under two years of age are most at risk. Otitis media can be caused by several different types of bacteria but is most commonly caused by the pneumococcus[24]. If

colonised by pneumococcal bacteria and the bacteria can spread from the nasopharynx into the middle ear cavity by way of the Eustachian tube it will lead to otitis media.

1.3.2 Pneumococcal pneumonia: Pneumonia is a lung disease that can begin quite suddenly with several symptoms including a severe chill, shortness of breath and chest pains. Bacterial pneumonia can be cause by several different bacteria, but pneumococcal pneumonia is the most common in adults. Children under 5 years of age, the elderly and people who are immunocompromised are most likely to experience serious complications if they are infected with pneumococcal pneumonia and elderly people who are infected are most likely to die from, this disease. Pneumococcal pneumonia is caused by the spread of bacteria from the nasopharynx to the lower respiratory tract. Pneumococcal infection triggers the accumulation of white blood cells and immune proteins in the alveoli. This accumulation of fluid makes it difficult for alveoli to function in the exchange of oxygen.

1.3.3 Bacteraemia: Blood is normally a sterile environment, so any bacterium detected is an abnormal event. The detection of viable bacteria in the blood is known as bacteraemia. Bacteria can enter the blood in several different ways including during surgery, through the introduction of a foreign object into the body or as a complication of severe infection. In some cases, bacteraemia can have no symptoms whereas in others fever and other symptoms can be experienced, with septic shock even being possible in some instances. Pneumococcal bacteraemia can lead to severe complications including meningitis if the bacteria manage to cross the blood brain barrier.

1.3.4 Meningitis: Meningitis is characterised by the inflammation of the lining around the brain and spinal cord. Bacterial meningitis is very severe, and meningitis caused by pneumococcal disease is one of the most common. Bacteria in the blood stream manage to cross the blood brain barrier and infect the cerebrospinal fluid (CSF) in the meninges. When this happens, bacteria can reproduce rapidly, causing severe inflammation and pressure on

the brain. This can lead to seizures and in the most severe cases can be fatal. Pneumococcal meningitis has a high mortality rate, particularly in vulnerable groups.

1.4 Epidemiology of *Streptococcus pneumoniae*

S. pneumoniae is one of the most important human pathogens worldwide causing pneumococcal diseases all around the world. It is a problem in children who live in developed as well as developing countries and it is estimated that more than a million children under the age of 5 die each year because of pneumococcal disease[25]. The advent of antibiotics has reduced the number of fatalities because of pneumococcal pneumonia, but it is estimated that there are still 500,000 cases of pneumonia each year in the USA alone. Pneumococcal pneumonia is also a severe disease of the elderly and people with other underlying illnesses. Pneumonia is estimated to affect 1% of the western population with *S. pneumoniae* being responsible for almost half the cases of community acquired pneumonia (CAP) in the UK[26].

Pneumococcal disease outbreaks tend to be higher in the winter month, perhaps because of an increase in viral respiratory infections that are seen during these months making people more vulnerable to pneumococcal infection.

S. pneumoniae is categorised based on its capsular polysaccharide. To date more than 90 different serotypes have been identified[20]. Serotypes are grouped together if they are antigenically related e.g. 9A, 9L, 9V whereas individual serotypes which share no antigenic relationship are given a different number[27]. Pneumococci are naturally transformable and as such can exchange genetic material between strains.

Serotype prevalence has changed over time. Since the introduction of Pneumococcal conjugate vaccine PCV7, the percentage of clinical isolates of vaccine serotypes (4, 6B, 9V, 14, 18C and 23F) have decreased and other non-serotype isolates have increased. In the

US, vaccine serotype isolates have reduced from 55% to 5% and the non-vaccine serotype 19A has increased substantially from 2% to 22%[28]. The release of PCV13 which contains additional serotypes including serotype 19A means that 19A is no longer increasing in prevalence but is still the most common clinical isolate in the US[28]. PCV7 was introduced as part of the routine vaccine schedule of many countries and a dramatic decrease in invasive pneumococcal disease has been observed within children vaccinated alongside a herd effect in children in other age groups[29]. But the increase in prevalence of non-vaccine serotypes still means invasive pneumococcal disease is a cause for concern[30].

Resistance to serotypes of *S. pneumoniae* has developed across the world dramatically in the last 30 years[31]. Serotype distribution differs geographically with antibiotic resistance also varying greatly. But it has been reported that serotypes 6B, 6A, 9V, 14, 15A, 19F, 19A and 23F show the highest rates of resistance to both erythromycin and penicillin[31]. The introduction of pneumococcal conjugate vaccine PCV7 was shown to reduce penicillin resistance rates[32, 33] but those serotypes not found in the vaccine, particularly 19A, have now been observed to have significant antibiotic resistance in multiple countries[30, 34].

There are several different risk factors that increase your likelihood of developing pneumococcal disease. Age is an important risk factor with Children under the age of 2 and adults over the age of 65 being high risk[35]. There are also many other important risk factors that can increase your chance if developing pneumococcal disease. People who smoke, who have asthma or COPD have been shown to be at increased risk in comparison to heathy individuals[36]. Alcoholism has also been associated with the development of severe pneumococcal disease with an estimated 50%of adult patients with pneumococcal pneumonia having a history of alcohol abuse[37]. People who suffer from HIV are 10-100 times more likely to develop pneumococcal pneumonia and bacteraemia compared to someone who isn't suffering from HIV[38].

1.5 Pneumococcal Virulence factors

1.5.1 Polysaccharide capsule

The polysaccharide capsule forms the outermost layer of *S. pneumoniae* cells and is approximately 200–400 nm thick. Pneumococcal serotypes are defined by their capsule and it is an essential virulence factor. Although non-encapsulated strains have been associated with superficial infections, invasive infections are almost always associated with encapsulated strains[39]. The capsule is important for several reasons. One of these is that within minutes of entering the nasal cavity, *S. pneumoniae* cells encounter mucus secretions. The expression of a capsule reduces entrapment in the mucus due to its negative charge, thereby allowing the pneumococcus to access the epithelial surfaces[40]. Un-encapsulated mutants are unable to escape the mucosal surface and are less likely to establish contact with the epithelial surface and establish colonisation. Conversely it has also been shown that strains of *S. pneumoniae* that have a larger amount of capsule are more likely to avoid entrapment within the mucous and more likely to evade initial clearance[41]. The capsule also acts as a defensive barrier for the bacteria by preventing the attachment of complement components and immunoglobulins to the bacterial cell wall[19]. This allows the bacteria to rapidly multiply before the host can clear it.

1.5.2 Pneumococcal surface proteins

More than 500 different surface proteins are present on *S. pneumoniae* which can be classified into four families. These families are choline binding proteins (CBP), lipoproteins, LPXTG proteins and non-classical surface proteins (NCSP) [42]. Both lipoproteins and LPXTG proteins can also be found on other gram-positive bacteria. These proteins have specific roles to play in different stages of pneumococcal infection. I have briefly outlined the roles of the different groups of pneumococcal surface proteins below.

1.5.3 Choline binding proteins

A notable group of surface proteins found on *S. pneumoniae* are the choline binding proteins (CBPs), a family of proteins that share the same structural organisation[43]. There are 12 CBPs bound to the choline of the pneumococcal cell wall and many are important virulence factors including PspA which is a protective antigen, LytA which is an autolysin and is responsible for the release of pneumolysin and CbpA which is an adhesin[42]. They are attached to the bacterial surface by the conserved choline binding site which binds non-covalently to the phosphorylcholine residues of techoic acid in the bacterial cell wall. They also have a biologically active site which projects from the surface of the bacteria. This group of proteins is characterised by the choline binding motif which is 2-10 repeats of 20 amino acids. Choline binding proteins exert their pathogenic effects either through host pathogen interactions or through enzymatic mechanisms[44].

1.5.4 Lipoproteins

Lipoproteins are universally expressed on bacterial surfaces and appear to be crucial for bacterial survival. Several lipoproteins can be found on *Streptococcus pneumonia, which* all contribute to its virulence. These include PsA, a divalent metal ion binding ABC transporter and sortases, SrtC and SrtD[45].

1.5.5 LPXTG proteins

This group of proteins is classified by a common mechanism of covalently binding to cell wall peptidoglycan. This mechanism is important for allowing the colonisation of the nasopharynx, with pneumococcal LPTXG adhesins able to bind to host sugar or protein molecules[46].

1.5.6 Non-classical surface proteins

Several surface proteins are expressed by *Streptococcus pneumonia* which cannot be classified into the categories of other surface proteins. These proteins lack classical features of other surface proteins and are thus categorised into a group of proteins called

non-classical surface proteins. 6 proteins can be found in this group, PavA, Eno, GADPH, 6PGD, HtrA and PGK.

1.5.7 Pneumolysin

Pneumolysin is a cytoplasmic enzyme belonging to a family of proteins called cholesterol-dependent cytolysins. It can cause cytolysis by forming pores in membranes that contain cholesterol. Upon binding to target membranes pneumolysin undergoes a conformational change which allows it to be inserted within the membrane and this form a pore complex. It is a 53 kDa pore forming toxin which is highly conserved amongst pneumococcal serotypes[47]. Pneumolysin is released by all serotypes of *S. pneumoniae* upon autolytic degradation of the bacteria. Autolysis involves the degradation of the cell wall by an autolysin. The main autolysin of *S. pneumoniae* is LytA (Nacetyl-muramoyl-1-alanine amidase). LytA cell wall hydrolysis allowing the pneumolysin, which is sequestered in the cytoplasm of the bacteria to be released and damage the host. Pneumolysin has numerous biological activities and acts in different ways depending on its lytic concentration. At sublytic concentrations a range of effects can be observed including activation of complement, induction of apoptosis and the triggering of pro-inflammatory responses by immune cells[47]. At higher lytic concentrations, the toxin forms pores in the membrane of cells leading to direct cellular and tissue damage. Isogenic mutants of *S. pneumoniae* that do not express have been shown to be cleared from the lungs following infection which suggest it has an important role to play in the virulence of the bacteria[48].

1.6 Host Immunity to *Streptococcus pneumoniae*

The immune system is an incredibly complex series of systems designed to be able to protect the host from a vast variety of pathogens and to protect any harm coming to the host. It is a complex array that must be able to target and kill invading pathogens, while being able to recognise self-antigen to prevent accidental damage to the host. The immune

system can be generally classified into two very broad, but also interconnecting parts based on their role during host immune defences. The adaptive immune system is based around T and B cell responses which can recognise a huge variety of antigens in an antigen specific manner, enabling the specific identification and subsequent elimination of pathogens. The adaptive immune system also provides long term protection against pathogens in the form of memory B and T cells which stay in the body and can offer immediate protection if they encounter the same antigen later. The innate immune system on the other hand, is responsible for providing an immediate but non-specific response and is an essential first line defence against invading pathogens.

1.6.1 Innate Immune Response

The innate immune system is the first line of defence against microbial infection. Innate responses are non-specific, using a limited number of receptors and secreted proteins to effectively distinguish between self and non-self-antigen. It is responsible for providing the initial defence against the pathogen and for inducing adaptive immune responses[49]. The importance of the innate immune response is clearly demonstrated as any defects in its components severely hamper the host's ability to fight infection, even when an intact adaptive immune response is present.

The first response is the production of antimicrobial enzymes and peptides in blood, then activation of the complement system. The second phase of the innate response is recognition of molecules called pathogen associated molecular patterns (PAMPs), which are found in microbes, not host cells. The recognition of PAMPs leads to a cascade of several effector mechanisms responsible for the clearance if the microbe.

Innate immune responses the pneumococcus includes the clearance of bacteria in the upper respiratory tract by secreted enzymes in the epithelial barrier, activation of the complement system leading to opsonisation of the bacteria followed by phagocytosis and

recognition of bacteria though pathogen associated molecular patterns (PAMPs)[49]. The role of each section of the innate response within pneumococcal infection is outlined below.

1.6.1.1 Breakdown of epithelium

Epithelial barriers are the host's first defence against invading pathogens. Failure of this innate immune response leads to dissemination of bacteria into areas where they can cause disease. For example, the breakdown of the respiratory epithelium allows bacteria to move into the lungs and can lead to pneumonia.

1.6.1.2 Complement

The complement system is a far reaching and essential part of the innate immune response. The complement system was first discovered in 1896 by Bordet and was named due to its ability to 'complement' the properties of antibody[50]. Complement is a very complex network of serum proteins, both plasma and membrane associated, that can elicit very efficient and well-regulated immune responses against pathogens[51].

It was thought that the complement system only plays a role in the innate immune responses and has no role to pay in adaptive immunity. Since the 1970's it has become increasingly obvious that the innate and adaptive immune responses cannot be so easily separated and that in fact they interconnect and overlap in many ways. This is demonstrated by the ability of complement to not only increase the effectiveness of the innate immune response but also in the way complement can influence T and B cell responses and so play a role in adaptive immunity[52].

The complement system is comprised of more than 30 proteins that are found in plasma and on cell surfaces. These proteins can be arrayed into protein cascades that begin with the identification of the invading pathogen and lead to either the induction of pro inflammatory mediators or in the opsonisation of the pathogen which ultimately leads to

the targeted destruction of the pathogen[53]. The activation of the complement system occurs through one of 3 pathways, the classical, lectin or alternative pathways. All 3 pathways culminate in three broad effector pathways which enable complement to successfully exert its effect in host defence[54]. The first of these effector pathways is the direct lysis of targeted surfaces by the formation of membrane attack complexes (MAC). A MAC is a pore of approximately 10nm diameter. The formation of the pores leads to the targeted lysis of the cell on which it is assembled. The second is the priming of the immune system to generate pro inflammatory cytokines and other pro inflammatory responses and the third is the opsonisation of pathogen surfaces by complement opsonin's (C4b, C3b, C3bi) which engage with complement receptors on the surface of phagocytes such as macrophages leading to the phagocytosis of the pathogen[54].

It is essential that the complement system be very tightly regulated to prevent unwanted damage to the host. The importance of complement as an innate immune defence against pneumococcal infection is well documented. People who have complement deficiencies are more at risk of contracting severe pneumococcal infection which is also more likely to recur throughout life[53]. Complement mediated phagocytosis is an essential innate response for the elimination of the pneumococcus. The complement system is triggered upon encountering the bacteria itself but is also activated when in the presence of specific pre-existing antibody against the bacteria because of previous infection[39].

1.6.1.3 Pattern Recognition Receptors (PRR)

Pattern recognition receptors act to initiate the recognition of microbes and bring about a response which targets the bacteria[55]. PRRs recognise regular patterns in microbial structure known at pathogen-associated molecular patterns (PAMPs) which are structures commonly found in many microbes but not in the hosts own cells[49]. Examples of PRRs are lipopolysaccharide (LPS), Toll-like receptors (TLRs), NOD-like receptors (NLRs) and

peptidoglycan.[49]. Recognition of microbes through PRRs leads to the recruitment of phagocytes such as neutrophils and macrophages to the site of infection. The phagocytes can clear the bacteria in several ways. Firstly, they can phagocytose the bacteria, but they also release a multitude of inflammatory mediators which cause the clearance of the bacteria. If the innate immune responses fail an acute inflammatory reaction is caused which can lead to host damage.

1.6.1.4 Toll-like receptors (TLR)

Toll-like receptors (TLRs) are a specific class of PRRs that play a key role within the innate immune system. 10 different TLRs have been identified in humans with TLR1, 2, 4, 5 and 6 being found on the cell surface in the plasma membrane and TLR 7, 8 and 9 residing inside the cell in endosomes[53]. TLRs bind different ligands and therefore become activated by different ligands. They are found on several different cell types including leukocytes, adaptive immune cells (T and B cells) and other non-immune cells such as epithelial and endothelial cells. As with other PRRs they recognize specific, conserved patterns within pathogens. Their specificity means they cannot easily be changed by evolution as they are recognizing molecules that are associated with a threat. They are highly specific to the conserved region, so they cannot mistake self-antigen for a pathogen. TLRs are responsible for activating immune cell responses upon recognition of these conserved repeats after a microbe has breached the physical barrier. Upon activation TLRs recruit adapter proteins which are responsible for mediating protein-protein interactions. The adapter proteins accumulate within the cytosol of the immune cell and are responsible for the triggering of signal pathways which leads to the activation of transcription factors. Ultimately this leads to the up regulation of genes that have a role to play in inflammatory responses such as cytokine production. If the TLR ligand is associated with bacteria, it could be phagocytosed, and its antigen presented to CD4+ T cells.

Pneumococcal components are recognised by different TLRs. Pneumococcal cell wall components including lipotechoic acid and lipoproteins are recognised by TLR2[55] whereas TLR9 can detect unmethylated CpG motifs within endosomal DNA. It has also been suggested that TLR2 and TLR9 are involved in the enhancement of phagocytosis of *S. pneumoniae* and intracellular killing of the bacteria by leukocytes.

The importance of TLR2, 9 and 4 in pneumococcal disease has been demonstrated using mouse models. As an example, during the late stages of pneumococcal colonization TLR2-/- mice show reduced clearance and have been shown to have reduced levels of IL-1β and IL6[56].

1.6.1.5 NOD-like receptors (NLR)

There are 22 members of the NLR family in humans with the majority being cytosolic proteins. NOD1 and NOD2 are responsible primarily for the activation of an NF-kB-dependent pro-inflammatory response. NOD1 acts by the recognition of peptidoglycan fragments that are mainly produced by gram negative bacteria whereas NOD2 is activated by peptidoglycan fragments of all bacteria regardless of gram status[57]. Other NLRs are responsible for the formation of protein complexes called inflammasomes. Examples include NLRP1 and NLRP4 and these are activated by a wide number of stimuli. NOD2 activation is involved in the detection of pneumococci that have been internalised by macrophages. During phagocytosis, S. pneumonia is degraded in macrophages leading to the delivery of peptidoglycan fragments into the cytosol of the host cell. This is involved in NOD2 activation.

1.6.2 Adaptive immune response

The adaptive immune response becomes very important if a pathogen can overcome the innate immune response. The innate immune response brings about a changed cellular environment and along with the continued replication of the pathogen this triggers the

adaptive immune response. Most pathogens can overcome the innate immune response, so the adaptive immune response is essential for the continued defence against them. The adaptive immune response can be separated into cellular immune response and antibody immune responses.

Naïve T cells are T cells that have not yet encountered their specific antigen. These naïve T cells circulate between blood and lymphoid tissues waiting to encounter its specific antigen. Upon antigen recognition, naïve T cells differentiate into effector T cells that are specialised to perform different roles. CD8+ T cells recognise pathogens presented by MHC class one molecules and naïve CD8+ T cells differentiate into cytotoxic T cells, which act to kill infected cells. CD4+ T cells on the other hand recognise pathogen presented by MHC class two molecules and can differentiate into several different effector CD4+ T cells each with a different role. CD4+ effector subsets that have currently been distinguished are T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and T follicular helper (Tfh) [58, 59]. Also, there are several regulatory subsets[60, 61]. Effector helper T cells act to activate macrophages and provide help to B cells to trigger the production of antibodies.

1.6.2.1 Cellular immunity

1.6.2.1.1 CD4+ T cells

CD4+ T helper cells are a critical component of the immune system and are important mediators of cellular immune responses. It was thought for many years that T helper cells existed in just 2 lineages, Th1 and Th2 cells[62]. These two groups were distinguished by the cytokines they released with Th1 cells producing IFN-γ and Th2 cells producing IL-4, IL-5 and IL-13. Recent analysis revealed that further subsets including Th17 cells also exist. IL17 has long been appreciated as a cytokine produced by T cells as protection against some pathogens[63]. In 2000, IL17A was shown to be released by a distinct population of T helper cells[64]. Further analysis revealed that T cells could differentiate into an IL17 producing

population of T helper cells independently of Th1 and Th2 cell development[65]. This established Th17 cells as a unique lineage of T helper cells.

CD4+ T cells are important as helpers for several immune cells including B cells, CD8 T cells and macrophages as well as having a role in the regulation of immune responses to control autoimmunity and adjust the magnitude and persistence of responses. Dysregulation of Th1, Th2 and Th17 cell differentiation can lead to severe immunopathology. If a Th1 response is too excessive it has been associated with numerous autoimmune diseases and inflammatory diseases[66] . Excessive Th2 responses are associated with allergic diseases and asthma[67]. It has been shown that Th17 responses are incredibly strong pro inflammatory responses and any reaction against self-antigen leads to severe autoimmune disease[68].

1.6.2.1.2 T cell activation

T cell development occurs in the thymus. Following T cell development, the mature naïve T cell will leave the thymus and circulate throughout the body. Some will continue to circulate while others will enter lymphoid tissue. Like all T cells, T helper cells express the T cell receptor (TCR) which consists of a constant and variable region. The variable region is responsible for determining which antigen the T cell responds to.

The TCR of CD4+ T cells has a high affinity for MHC class 2 which is found on the cell surface of Antigen Presenting Cells (APCs) such as dendritic cells and macrophages. During an immune response an APC such as a dendritic cell can endocytose the antigen which is then processed into peptide fragments before forming a complex with MHC class 2[69]. The APC will travel to the site of infection and is able to present the antigen bound to the MHC class 2 to CD4+ T cells which express the specific TCR against the peptide- MHC complex which causes the T cell to activate[70].

Upon recognition of antigen bound to an APC by T helper cells, The TCR in complex with CD3 binds to the peptide-MHC complex. CD4 acts as a co-receptor which specifically recognises MHC class 2 and this also binds to the MHC class 2 molecule, but in a different section[71]. These interactions allow the two cells to be brought closely together which allows intracellular kinases present of the TCR, CD3 and CD4 to phosphorylate each other and become active. This activates intracellular T helper cell pathways which are commonly referred to as signal 1[72]. When the same antigen is then subsequently encountered by memory T cells these same TCR pathways are activated.

Upon completion of signal 1, a second signal is given known as signal 2. Signal 2 is a protective signal which prevents the T cell from responding to self-antigen. If signal 2 is not activated during the initial exposure of the T cell to antigen, the T cell presumes it is auto-reactive and becomes anergic[73]. This means the cell will not respond to any antigen in the future even if both signal 1 and signal 2 are received. Signal 2 involves an interaction between CD28 on the CD4+ T cell and CD80 and CD86 on the APC[74]. CD80 and CD86 are co-stimulatory molecules which activate the CD28 receptor.

After activation using signal 1 and signal 2, the T cell is then able to proliferate. Proliferation occurs by the release of IL2 which acts upon T cells in an autocrine fashion. The IL2 can bind the T helper cell that is secreting it or other, neighbouring T helper cells via the IL2 receptor and thus proliferation and clonal expansion of the T helper cells is driven[75]. A T helper cell, which has been both activated and proliferated then, becomes a T helper 0 (Th0) cell that is able to secrete IL2, IL4 and IFN gamma. Th0 cells will then be differentiated into Th1, Th2 or Th17 cells depending on the cytokine environment[76].

The activation of T cells has to be tightly controlled to prevent the accumulation of a massive amount of activated T cells. This is achieved through a negative feedback loop involving CTLA-4 which acts to inhibit intracellular signalling[77]. CTLA-4 is a cell surface

protein and is expressed by the T cell upon activation. CTLA-4 binds to CD80 and CD86 on the surface of the antigen-presenting cell with a much higher affinity than CD28, the co-receptor protein involved in signal 2 production. When CTLA-4 is bound to CD80 and CD86 the activation of T cells is halted[78].

1.6.2.2 Effector T cells

1.6.2.2.1 Th1/Th2 cells

Th1 cells are one of the lineages of T helper cells and they promote a cellular response to protect the host against intracellular pathogens. Th1 cells are characterised by the secretion of IFN gamma, IL2, IL10 and TNF alpha and beta[79]. Th1 cells cause the activation of macrophages and the proliferation of cytotoxic T cells which ultimately leads to the phagocytosis and destruction of the invading pathogen. Their cytokine profile is one of the main ways of differentiating Th1 cells from other T helper cell subsets, but they can also be distinguished by their specific cell surface receptors which include IFN-γ R2, CCR5, and CXCR3 and IL-12 R β2[80]. These receptors are essential for interaction with the cytokines that drive the differentiation of Th1 cells from naïve T cells including IL-27, IL-12 and IFN gamma. Th1 cells are critical for the clearance of intracellular pathogens but their over expression has been shown to be associated with autoimmune diseases including inflammatory bowel disease and multiple sclerosis[79].

The differentiation of naïve T cells to Th1 cells begins with IL27 signalling. This induces STAT1- dependent expression of T-bet which is a Th1 specific transcription factor and is the master regulator of Th1 cells[81]. T-bet promotes the expression of IFN-γ and IL-12 R β2. Il12 R β2 is then able to heterodimerize with IL-12 R β1 and form a functional IL12 receptor complex. This leads to the increase in IFN-γ expression[58].

Th2 cells offer protection against extracellular pathogens by mediating the activation and maintenance of antibody mediate immune responses. Th2 cells produce numerous cytokines including IL-4, IL-5, IL-6, IL-9, IL-13 and IL-25 which cause antibody production, activation of eosinophils and inhibit macrophage function, making their protective responses phagocyte independent[59]. The cytokines produced by Th2 cells also act to counteract Th1 cell responses.

The effects of Th2 cell cytokines is seen on many different cell types throughout the body. They can stimulate and recruit specialised immune cells such as basophils and eosinophils to the site of infection or in response to allergic or toxic antigen[82]. This causes the induction of mucous production and airway hyper responsiveness. Th2 cells are also responsible for the class switching of B cells to IgE. When over responsiveness of Th2 cells occur, allergies become exacerbated and Th2 cells have also been implicated in the onset of asthma and other allergic inflammatory diseases[67].

Th1 cells and Th2 cells have a mutually exclusive relationship. When one is present the other isn't present. This is because they each secrete cytokines that inhibit the activation and differentiation of the other. Secretion of IFN-y by Th1 cells inhibits development of Th2 cells and secretion of IL-4 and IL-10 by Th2 cells inhibits Th1 cell development.

1.6.2.2.2 Th17 cells

Recently a new class of T helper cell has been discovered: Th17 cells. Th17 cells exhibit effector functions that are distinct from Th1 and Th2 cells. The characteristic cytokines found upon Th17 activation are IL-17A, IL-17F, IL-22 and IL-23. These effector cytokines can be produced very quickly upon infection at mucosal sites. IL-17A and IL-17F act through the IL-17 receptor (IL-17R) which is composed of two subunits IL-17RA and IL-17RC. In doing so they promote the induction of pro-inflammatory cytokines and chemokines[83]. The IL-17

receptor is found ubiquitously so the release of IL-17 can cause the stimulation of a variety of cell types.

IL-17A and IL-17F are the best-defined cytokines secreted by Th17 cells and are important in mediating and inducing pro-inflammatory responses to bacteria. It is responsible for the recruitment of neutrophils leading to the clearance of bacteria. IL-17 acts on epithelial and endothelial cells as well as a subset of monocytes to produce pro inflammatory cytokines and chemokines and this leads to the rapid recruitment of neutrophils[84].

1.6.2.2.2.1 Th17 cells and pneumococcal infection

Th17 cells are shown to be important against several mucosal infections. They also appear to have a particularly important role in pneumococcal clearance [85, 86]. Th17 cells are responsible for the clearance of the pneumococcus by the recruitment of neutrophils and macrophages, and Treg cells act to regulate the Th17 cell response[87, 88]. This regulation is important to prevent an uncontrolled influx of leukocytes which could cause tissue damage and could allow the bacteria to disseminate in to the blood stream. However, studies suggest that the pneumococcus, like many other mucosal pathogens, can manipulate this system and induce Treg cells, which mean it is not cleared from the nasopharynx, and a sustained carriage state is reached.

Several studies have shown that Th17 cells are important in the clearance of the pneumococcus from the nasopharynx in order to prevent colonisation[89]. Upon stimulation by anti-pneumococcal antigens, CD4+ T cells in the tonsils undergo a greater response than those in the periphery. This suggests that CD4+ T cells are sequestered in the tonsil which makes sense, as from this position they would be more likely to encounter pneumococcal antigen and be able to quickly mount an immune response to clear infection[90]. A study by Lu et al showed that IL-17 was produced upon in vitro stimulation with pneumococcal antigens and that this enhanced level of IL-17 led to a greater level of phagocytic killing[86].

Several other studies have also shown increases in IL-17 production by CD4 T cells upon stimulation with pneumococcal antigens[91].

1.7 Regulatory T cells

1.7.1 Classification

The immune system must be tightly controlled to prevent undesirable side effects which could be caused by immune cells. An uncontrolled immune response can lead to severe host tissue inflammation which can cause more harm than the infection. Regulatory T cells are a fundamental component of a healthy immune system and have been implicated as having a role to play during microbial infection, allergy, autoimmunity, tumours and transplantation[92]. They act to balance effector responses to prevent pro-inflammatory responses to self-antigen. They have also been implicated as important in mucosal immunity. It is well documented that a deficiency in Treg frequency or number, or a defect in their function can lead to inflammation and/or autoimmune disease. Over the years several populations of CD4+ Treg cells have been identified. These include Foxp3+ regulatory T cells, Th3 cells and Type 1 regulatory T cells (Tr1)[93].

A naturally occurring suppressive subset of T cells was first described in the 1970's[94]. However due to a series of negative results the hypothesis was rejected. The "suppressor T cell" hypothesis was revived by studies showing that a subset of CD4+ T cells which express CD25 are essential for control of autoreactive T cells in vivo[95]. It was shown that depletion of CD25+ T cells in adult mice led to the development of various autoimmune diseases including gastritis[95]. This work led to the classification of a naturally occurring CD4+CD25+ subset of Treg cells. Recently, the discovery of a transcription factor which is expressed on this cell type, the forkhead-winged helix transcription factor forkhead box P3 (FoxP3), has allowed this cell type to be distinguished from other CD4+CD25+ T cells[96].

1.7.2 Foxp3+ Treg cells

Foxp3+ Treg cells have an important role in the prevention of auto immune disease and in the maintenance of immune tolerance. Dysfunction of Foxp3+ Treg cells through, for example a mutation in Foxp3, can cause fatal autoimmune disease. Foxp3+ Treg cells can be broadly classified into two subsets, naive Tregs (nTregs) and inducible Tregs (iTregs). nTregs mature in the thymus and make up 5-10% of the peripheral CD4+ T cell population[97]. nTregs are thought to be the population of Treg cells responsible for maintaining self-tolerance[98]. iTregs are thought to acquire their suppressor activity through activation of CD4+CD25-FoxP3- T cells in the periphery upon stimulation by an antigen. Activation of iTregs occurs in the presence of TGF-β1[99]. It is thought that iTregs could have a particularly important role to play at mucosal surfaces where there is an abundance of TGF-β1. TGF-β1 isn't required for the differentiation of nTregs but does seem important in promoting their survival in the periphery[99].

1.7.2.1 Identification of Foxp3+ Treg cells

As well as the presence of Foxp3 there are several characteristic markers of Foxp3+ Tregs. Foxp3+ Tregs are CD4+CD25+CD127^{low} and looking for these markers allows the identification of this cell type[100]. Naïve and inducible Tregs can be distinguished by the presence or absence of CD45RO and CD45RA. nTregs are characterised by their expression of CD45RA and low levels of FoxP3. iTregs are characterised by the expression of CD45RO and high levels of FoxP3[90].

1.7.2.2 Mechanisms of suppression

Foxp3+ Treg cells use the αβTCR for antigen recognition and are restricted by MHC-II molecules as with other CD4+ T cells[101]. It is thought that there are 2 mechanisms by which Treg cells exert their suppressive effects. The contact dependent method involves CTLA-4

on the Treg cell and CD80/86 on the effector T cell[102]. CTLA-4 is expressed at high levels on CD4+CD25+ Treg cells and it is thought that the CTLA-4 interacts with the CD80/86 on the effector T cell to decrease effecter T cell function. Immunosuppressive cytokines are also important in Treg associated suppression. IL-10 and TGF-β released by Treg cells are responsible for down regulating MHC II and co-stimulatory molecules on dendritic cells which affects their ability to present antigens and so stops the activation of CD4+ T cells[102].

1.7.3 Type 1 regulatory T cells (Tr1)

Type 1 regulatory T (Tr1) cells are an inducible subset of regulatory T cells that play a role in promoting and maintaining tolerance[103]. One of the main ways of identifying Tr1 cells has been through their cytokine profile. Tr1 cells are characterised mainly by high expression of IL-10. They also express minimal amounts of IL-4 and IL-17 which allows them to be distinguished from Th2 and Th17 cells respectively which express significantly higher levels of their respective cytokine[98]. They express a low amount of IL-2 but again the levels are not significant, so they can be distinguished from Th1 cells which express these cytokines in much higher level[98].

The original study that led to the discovery of IL-10 producing Tr1 cells began in the late eighties[104]. Tr1 cells were discovered in the peripheral blood of patients with severe combined immunodeficiency (SCID) with long term mixed chimerism after HLA-mismatched fetal liver hematopoietic stem cell transplant (HSCT) [104]. The SCID patients developed long term tolerance to stem cell allografts which suggested there was something regulating immune cells. Isolated cells did not show the same cytokine profile as Th1 or Th2 cells which suggested a unique cell type was responsible for the immune cell regulation. A few years later Groux et al demonstrated that Tr1 cells, producing high amounts of IL10, are a distinct subset of CD4+ T cells that are antigen specific and show strong immunosuppressive activity[105].

Tr1 cells can control the activation of naive and memory T cells and suppress Th1 and Th2 cell mediated immune responses. The suppressive effects of Tr1 cells are reversed by blocking antibodies against IL-10, showing that the inhibitory capacity of Tr1 cells is mainly mediated through production of IL-10. They also kill myeloid cells by the secretion of Granzyme B[98].

1.7.3.1 Identification of Tr1 cells

Tr1 cells are induced when CD4+ T cells are activated by antigen in the presence of IL-10[106]. It had been difficult to distinguish these cells from other cell types, relying on characterisation using its unique cytokine profile. Tr1 cells are distinguished from Th1 cells by expression of minimal IL4 and down regulation of GATA3 expression[1]. Tr1 cells lack IL17 and express low levels of RORC which distinguishes them from TH17 cells. Low levels of IL2 and IFN gamma which distinguishes them from CD25+Foxp3+ Treg cells. It has recently been shown that there are cell surface markers characteristic of Tr1 cells which allow for them to be differentiated from other regulatory T cells[1]. Tr1 cells are CD4+CD49b+LAG3+[1]. This ability to differentiate Tr1 cells from other cells means further study of this cell type is possible.

1.7.3.1.1 IL-10

IL-10 is an immunomodulatory cytokine that plays an essential role in regulation of the immune system in a variety of ways. It is responsible controlling inflammatory responses by down regulating inflammatory cytokines, induces tolerance and is important in the down regulation of other immune responses[107]. IL10 is indispensable for driving the differentiation of Tr1 cells but other cytokines are also involved.

During infection IL10 inhibits the activity of a variety of different cell types including Th1 cells and macrophages. These cells are essential for the clearance of pathogens but can also

be responsible for causing tissue damage which is why they must be regulated[108]. For this reason, IL10 can be responsible for inhibiting clearance of pathogens and preventing inflammation associated immunopathology.

IL10 was first described as a cytokine produced by Th2 cells which acted to inhibit Th1 cell responses, but it is now known that IL10 is produced by several other cell types including macrophages, dendritic cells and various subsets of CD4+ and CD8+ T cells[109]. IL10 regulates T cell responses with many of the effects being indirect and occurring via the direct effect of IL10 on macrophages[108]. IL10 inhibits MHC class 2 and costimulatory molecule B7-1/B7-2 expression on macrophages which limits their production of pro inflammatory cytokines and chemokines[110]. IL10 can also directly act on CD4 T cells, inhibiting their proliferation and the production of numerous cytokines including IL2, IL4 and IL5[110]. This allows IL10 to directly regulate Th1 and Th2 responses.

1.7.3.1.2 CD49b/LAG-3

LAG-3 is a protein antigen expressed on T cells and NK cells after cell activation. It is a CD antigen given the name CD223[111]. The expression of LAG3 on the surface of activated T cells is upregulated by IL2, IL7 and IL12 and its expression leads to the production of IFN-γ. LAG-3 has also been shown to be expressed on activated B cells but not when the B cell is activated solely by a TLR agonist[112]. LAG-3 expression on B cells could therefor serve as a marker for B cells that have been activated by T cells.

LAG-3 is a multifunctional protein and can affect a variety of different cell types. LAG 3 is a ligand for MHC class 2 and the interaction between LAG3 and MHC class 2 controls CD4+ T cell responses and leads to the down regulation of antigen specific CD4+ T cell proliferation and cytokine secretion[113].

LAG-3 is cleaved into 2 fragments which affects its function. A 54-kDa fragment, which includes the extracellular domain and a 16-kDa peptide containing the transmembrane and cytoplasmic domains. The 54 kDa fragment is released from the cell surface as soluble LAG-3 and it appears to cause the increase in T cell activation[114].

LAG-3 has been shown to be important for maximal function of regulatory T cells. Antibodies against LAG-3 have been shown to inhibit suppression by induced Treg cells both in vitro and in vivo[115]. LAG-3 knockout mice have been shown to have regulatory T cells with reduced regulatory function[115].

CD49b (cluster of differentiation 49b) is a protein encoded by the CD49b gene. CD49b is the α2 integrin subunit of very late activation antigen 2 (VLA-2)[1]. Integrins are integral membrane glycoproteins composed of an alpha and a beta subunit and are found on a variety of cell types including T cells and NK cells[116].

1.8 Role of regulatory T cells in pneumococcal disease

Nasopharyngeal colonisation is very common in young children before natural immunity can develop. In the UK colonisation in children less than 3 years of age is between 40-50%[117] but in African children this can reach 90-100%[118, 119]. By the age of 3 most children have developed natural immunity against specific pneumococcal antigens. This natural immunity is responsible for the clearance of the pneumococcus from the nasopharynx before carriage status can be achieved. However, despite this natural immunity carriage can still occur throughout life and it is thought that Treg cells could have a role in this.

Due to the success of vaccines targeted to the polysaccharide capsule of the pneumococcus, it has classically been thought that protection against it was due to an antibody-based response[120]. However, more and more publications are now suggesting an important role for CD4+ T cell immunity in protection against the pneumococcus. MHC-II

knockout mice which lack the ability to induce cell-mediated immunity through the presentation of antigen show prolonged carriage [120] suggesting CD4+ T cells have an important role to play in pneumococcal carriage rather than antibody-based responses.

In a recent study by Zhang et al, adenoidal cells from children who tested positive for pneumococcal carriage were found to contain higher numbers of Foxp3+ Treg cells when stimulated with pneumococcal whole cell antigen than those taken from children that tested negative[90]. The Treg cells present are of a memory phenotype suggesting they have been induced by a previous colonisation event. This association between carriage state and the increased number of Treg cells in the nasopharynx suggests that colonisation with the pneumococcus leads to the promotion of pneumococcus specific Treg cells. As well as this, the depletion of these cells before stimulation with whole cell antigen led to an increase in CD4+ T cell proliferation and the re-introduction of the Treg cells suppressed this effect[121]. This suggests that these memory Treg cells present in the nasopharynx have a potent inhibitory effect on CD4 T cell proliferation and may contribute to the persistence of carriage seen in young children. The next step in this story is to try and work out what specific component or components are responsible for the induction of Treg cells in the nasopharynx.

There have been several studies that have looked at different pneumococcal components to see how they affect colonisation and how they alter CD4+ T cell responses. The aim of this research is to try and identify components which could be used to inform the design of a novel vaccination strategy. Ideally a mucosal vaccine would contain several different protein antigens as this would increase its effectiveness across all pneumococcal serotypes. Protein antigens that have been looked at include pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), Choline binding protein A (CbpA) and pneumolysin[47].

Intranasal immunisation of mice using killed whole cell pneumococci and cholera toxin adjuvant conferred protection against experimental colonisation. This protection was dependent on CD4+ T cells[120]. In addition to this, immunisation with cholera toxin B subunit together with pneumococcal surface adhesin A (PsaA) was enough to provide protection against colonisation with the pneumococcus[122]. This suggests PsaA could be important for colonisation. It has also been demonstrated that a fusion conjugate containing cell wall polysaccharide, Pneumolysin and PsaA delivered alongside cholera toxin nasally protected mice against experimental colonisation[123].

Another study showed that D39 deficient in PspA and the D39 deficient in PspC (CbpA) have a reduced capacity to colonise the nasopharynx[124]. A Pneumolysin deficient mutant also had a reduced capacity to colonise the nasopharynx although this was to a lesser extent[125].

Pneumolysin has been shown to be essential for maintaining long term asymptomatic carriage in the nasopharynx[126]. Upon administration of a Wild type WT strain carriage was maintained for 28 days whereas after administration of PLN-A (pneumolysin deficient mutant) carriage was cleared between 7-14 days after infection. Another study has shown that stimulation of adenoidal MNCs with Ply or F433 leads to proliferation of CD4 T cells[85].

From these studies it is suggested that a variety of pneumococcal proteins appear to play a role in the colonisation of the nasopharynx. The next step in the story is to work out how they can do this.

Several papers have suggested that pneumococcal components can suppress allergic airways disease and are able to do this through the induction of Treg cells[127]. Using a mouse model of allergic airways disease, Thorburn et al identified that type 3 polysaccharide (T3P) and Pneumolysin when administered together caused an increase in Treg cell numbers in the lungs when compared to a control group[127].

There is overwhelming evidence showing that Treg cells and Th17 cells have important roles to play in many mucosal infections. These cell populations have a key role in the outcome of these infections but whether they have a positive or negative effect appears to depend on the pathogen. With regards to pneumococcal infection, Treg cells appear to be important for carriage of the pneumococcus in the nasopharynx and have been shown to be responsible for supressing CD4+ T cell responses which would lead to the clearance of the bacteria from its site of colonisation. Further research is necessary to look at which specific components and what mechanisms are used to induce antigen specific Treg cells in the nasopharynx of individuals.

1.9 Role of regulatory T cells in pathogen persistence

Studies[90, 128, 129]suggest that the pneumococcus, like many other mucosal pathogens, can manipulate the immune system and induce Treg cells, allowing the pathogen to persist and not be cleared from the nasopharynx. This allows the carriage of the bacteria and its spread to other individuals in the community.

Foxp3+ Treg cells are essential for protecting against inflammation mediated tissue damage but they also suppress immune responses which are necessary for the clearance of the bacteria to prevent pathogen persistence[130]. Effector T cells, in particular Th17 cells, are responsible for the clearance of the bacteria but if with no regulation, they would cause severe tissue damage. Treg cells can inhibit the action of important adaptive immune cells including macrophages and T and B lymphocytes which promotes an environment prefect for the carriage of the bacteria. This is why an equilibrium must be reached. To allow Treg numbers to promote pathogen clearance while at the same time protecting from inflammation associated damage caused by uncontrolled effector cell proliferation and action[130].

Treg cells can affect a wide range of host immune cells including CD4+ and CD8+ T cells, B cells and antigen presenting cells such as dendritic cells and macrophages[130]. Treg cells act by inhibiting proliferation and cytokines of effector cells. They inhibit the production of antibody and reduce the ability of APCs to stimulate T cell responses by inhibiting the expression of co-stimulatory molecules and inflammatory cytokines involved in APC responses[130].

1.9.1 Bacteria

IL-17A and IL-22 produced by Th17 cells are important against *Klebsiella pneumoniae* infection. *K. pneumoniae* is a gram negative opportunistic pathogen that causes acute pneumonia[131]. During infection there are measurable levels of IL-17A and IL-22 which act to recruit neutrophils to the lungs and decrease the bacterial burden. IL-22 has been shown to have a role in preventing the dissemination of bacteria from the lungs to the spleen where it can do even more damage. So the IL-22 is important for the augmentation of barrier defences[131].

Mycobacterium tuberculosis is the pathogen that causes tuberculosis. It is estimated that approximately one third of the world population is infected *with M. tuberculosis* and people infected have a high morbidity and mortality. During *M. tuberculosis* infection the bacteria is phagocytosed by macrophages but the cell wall prevents fusion with the lysosome[131]. This means the bacterium is protected and can divide. Th1 cells are essential during *M. tuberculosis* infection as they directly act to get rid of the bacteria. Th17 cells are also important, although in an indirect way, as they are responsible for the recruitment of Th1 cells[132]. So, in this case the role of Th17 cells is not a primary role but is nevertheless important and as such is being targeted in vaccine development strategies. Studies[133, 134] have shown that during *M. tuberculosis* infection there are a high number of FoxP3+ cells in

the lungs of mice and humans which suggests that Treg cells have a role to play in the persistent nature of the infection.

Helicobacter pylori is a gram negative pathogen that infects the gastric mucosa[84]. It is associated with the development of ulcers and cancers. IL-17A produced by Th17 cells is essential to the inflammation that is seen during *H. pylori* infection. Th1, Th17 and Treg cells all have a role to play during *H. pylori* infection and the outcome of the infection depends on how these cells are balanced[135]. There are several papers documenting the relative importance of Th17 and Th1 cell responses to *H. pylori* infection. Some papers argue that IFN-γ produced by Th1 cells is implicated in the pathology associated with the infection, whereas others show that IL-17 neutralization reduces gastric inflammation associated with this infection implicating Th17 cells as the main subset associated with inflammation. A study by Flach et al compared the importance of four different cytokines and showed that only the neutralisation of IL-17 was associated with an increase in *H. pylori* colonisation[136] . This strongly suggests that Th17 cells are more important for protection against *H. pylori* than Th1 cells. *H.pylori* infection can persist for decades in the gut of individuals despite a robust immune response from the host. It has been shown that this is since antigen specific CD4+CD25+ FoxP3+ Treg cells accumulate in the gastric mucosa of people who are infected with *H. pylori*. The bacterium can actively skew the immune response towards producing Treg cells and thereby suppresses Th17 driven immunity[137]. In doing so the inflammation associated with the infection is prevented but bacteria are persisting in the gut. So, the Treg response protects the gut against exaggerated inflammation but in turn allows the bacterial burden to increase. This can lead to chronic infection and can be the cause of the development of gastric cancer[138]. This fine balance would need to be considered by anyone hoping to produce a vaccine against this infection as by skewing the immune response towards one or the other subsets of T cells could have disastrous outcomes.

Bordetella pertussis is the cause of severe respiratory disease which can often be complicated by secondary infections which can lead to death in young children. Challenge with *B. pertussis* induces both Th1 and Th17 cell responses. IFN-γ is produced to coincide with the peak of infection and IL-17 is detected before IFN-γ and continues until the infection is cleared[139]. *B. pertussis* specific Th1 cells are associated with the recovery from this infection but during the acute phase of infection the antigen specific Th1 cell response is suppressed by pathogen specific Treg cells. This allows persistence of infection. However, the absence of a strong Treg cell response led to an increase in inflammatory cytokines and extensive damage to lung tissue. This suggests that perhaps Treg cell induction is important to prevent the over enthusiastic Th1 cell response. Once again this highlights the delicate balance between T helper cells and Treg cells.

1.9.2 Fungi

Candida albicans causes oropharyngeal candidiasis (OPC) which is a biofilm-like infection of the oral mucosa. IL-17 mediated recruitment of neutrophils is important for host defence against *C. albicans*. When mice that are deficient in the IL-17 receptor are infected with *C. albicans* there is decreased survival seen when compared to a wild type control showing that Th17 cells are important during infection[140]. During *C. albicans* infection there is an unexpected interplay between Th17 cells and Treg cells with both seeming to be important for providing protective immunity. Mice which lack T and B cell responses are very susceptible to OPC infection. Transfer of Th17 cells to these mice isn't enough to protect against infection but co transfer of Treg cells and Th17 cells confers protection through an enhancement of Th17 cell responses[140]. So, the Treg cells appear to be boosting rather than suppressing the Th17 cell response.

1.9.3 Virus

During *Herpes Simplex Virus 2* (HSV-2) infection, which causes genital herpes, the removal of Treg cells causes an increase in virus associated inflammation and increases mortality. The viral load is also dramatically increased upon the removal of Treg cells[141]. Infection of the cornea due to *Herpes simplex virus 1* (HSV-1) induces early induction of IL-17 and IL-23. Knockout mice which lack the IL-17 receptor see reduced recruitment of neutrophils during early infection which leads to damage to the cornea following infection with HSV-1.

1.9.4 Parasite

Plasmodium falciparum is the parasite that causes human malaria. During malarial infection increased numbers of Treg cells have been found in both human and mouse malaria infection. In mouse models of malaria, it was shown that Tregs act to supress CD4+ T cell responses and in doing so cause in increase in the parasite load. In human infection it has also been shown that higher numbers of Treg cells are associated with increased parasite load and development of infection. A study conducted among Gambian children who had malaria and those who were healthy (control) showed that Treg cells were unable to control inflammation during acute and severe infection which suggests they may be overwhelmed by the parasite[142]. Later in infection Treg cells may be important in down regulating inflammatory responses when the parasite is already well on its way to being cleared by the immune response. But suppression too early in infection would hamper the responses needed to clear the infection and allow the parasite burden to increase once more.

Toxoplasma gondii is an obligate intracellular parasite that causes toxoplasmosis. In a mouse model, expansion of Treg cells protects the host against fatal immune pathology. Following infection there is a natural collapse in Treg cell numbers which allows a

potentially fatal infection to take hold. Increasing the number of Treg cells prevents this from happening.[143]

1.10 Antibody responses against *Streptococcus pneumoniae*

1.10.1 B cells

B cells develop from stem cells that originate in bone marrow. B cell development occurs in several stages, each marked by the expression of different gene patterns and different arrangements of the immunoglobulin H chain and L chain gene loci[144]. B cells can either undergo positive or negative selection. Positive selection occurs through antigen dependent signalling involving the pre- B cell receptor and the B cell receptor. If the B cell receptors do not bind to their ligand, B cell development ceases. Negative selection occurs when the BCR binds to self-antigen and allows for tolerance of self-antigen[145].

Immature B cells move from the bone marrow to the spleen where they pass through 2 transitional stages of development before undergoing the final stage of development. Upon entry to the spleen B cells are considered T1 B cells (transitional stage 1) but whilst in the spleen they transition to T2 B cells (transitional stage 2). T2B cells differentiate into either follicular B cells or marginal zone B cells depending on signals received through the BCR and other receptors. Upon differentiation they are now considered to be full mature naïve B cells[146].

Activation of B cells occurs in secondary lymphoid organs such as the spleen and lymph nodes. In the secondary lymphoid organs, the B cells receive constant supply of antigen via circulating lymph. B cell activation begins with the binding of antigen to the BCR. The antigen can be free antigen or antigen presented by APCS such as dendritic cells or macrophages. Activation of B cells is enhanced through the activity of another surface receptor on B cells, CD21. CD21 forms a complex with surface protein CD19 and CD81

which is collectively known as the B cell co-receptor complex[145]. If an antigen that is tagged with C3 (complement component) the C3 fragments ligates with the CD21 which transduces a signal through CD19 and CD81, lowering the activation threshold of the cell and thus enhancing B cell activation[147].

T cell dependent antigens as the name suggests are antigens that induce B cell antibody responses with the help of T cells. B cell responses to these antigens take several days but the antibodies produced have a higher affinity and functional versatility than B cells responses generated from T cell independent activation[148].

When a T cell dependent antigen binds the BCR, it is endocytosed, degraded and presented to T cells as fragments of peptide in a complex with MHC class 2. T helper cells that are specific to the same antigen and thus were activated by the same antigen recognise and bind the MHC class 2-peptide complex through the TCR. Following the binding of the TCR to the MHC class 2-peptide complex, a surface protein, CD40L and cytokines including IL4 and IL21 are expressed. CD40L is a co-stimulatory factor for B cell activation and binds the B cell surface receptor CD40 leading to B cell proliferation as well as sustaining T cell growth and proliferation. Cytokines derived from T cells bind to cytokine receptors on B cells also promote B cell proliferation. Upon receipt of these signals a B cell is considered active[149].

Upon activation B cells undergo a 2-step differentiation process which yield both short lived plasmablasts, responsible for immediate protection and long-lived plasma cells and memory B cells which allow protection for a much longer period. The first step is called the extrafollicular response and occurs within the secondary lymphoid tissue, outside the lymphoid follicles. During this step B cells can proliferate, may switch between classes of immunoglobulin and differentiate into plasmablasts which produce antibodies for immediate protection but are weak[150]. During the second step, B cells migrate into the lymphoid follicles and form a germinal centre, a specialised area where B cells undergo

extensive proliferation and immunoglobulin class switching. B cells are helped by T cells (mainly Tfh cells) which are localised within the germinal centre and the result is the production of high affinity memory B cells and long-lived plasma cells. The plasma cells secrete large amounts of antibody and generally localise within the bone marrow[150].

T cell independent antigens such as polysaccharide and unmethylated CPG DNA can induce a humoral response without the need for T helper cell interactions. The B cell response to T cell independent antigen is very rapid but the antibodies produced tend to be of a lower affinity and have less functional versatility compared to antibodies produced by T cell dependent activation[151].

Additional signals are still required for B cells to complete activation, but they do not receive them from T cells. Instead, B cells are activated by TLR's or repeating epitopes on bacterial cell walls. B cells activated by these antigens further proliferate in secondary lymphoid tissue but not in lymphoid follicles and no germinal centre is formed[152].

Memory B cells are activated upon the binding of their target antigen. Upon binding of the antigen, endocytosis occurs to allow the memory B cell to engulf the antigen. The antigen is degraded before peptide fragments form a complex with MHC class 2 which can then be presented to T cells. T helper cells which recognise the same antigen as the memory B cell, recognise the MHC class 2-peptide complex and bind to them leading to a signal cascade that ends with B cell activation and proliferation[51].

1.10.2 B cells and *Streptococcus pneumoniae*

When pneumococcal capsular polysaccharide activates naïve B cells they are able to differentiate into IgM+ memory B cells which can produce IgM specific to the pneumococcal antigen without any additional T cell help being required[153]. During latter stages of infection pneumococcal specific IgM+ B cells can differentiate into pneumococcal

specific IgG+ or IgA+ memory B cells[126]. IgA is recognised as being important against pneumococcal infection as it is commonly found at mucosal sites such as the nasopharynx, and after mucosal infection, IgA is detected in the nasopharynx[126]. In both mouse models and clinical studies, a deficiency in IgA lead to bacterial persistence in the nasopharynx and the increased likelihood of recurrent infection[154, 155].

IgM+ B cells are also an important humoral defence against pneumococcal infection. It has consistently been shown that patients with low levels of IgM+ memory B cells are more prone to develop recurrent respiratory infections[156]. Mouse models have shown that when B cells that produce IgM are depleted, response against pneumococcal polysaccharide are severely impaired[157]. These studies, and more not mentioned here, suggest IgM and IgM+ memory B cells play a key part in humoral immune responses against pneumococcus.

1.11 Mucosal immune system

The mucosal immune system is part of the adaptive immune system and is responsible for activating antigen specific T and B cells. The mucosal immune system is commonly referred to as the MALT (mucosal associated lymphoid tissue) and MALTs are distributed widely throughout the mucosal surfaces of the body. They are named based on their location for examples gut associated lymphoid tissue (GALT) and nasopharynx associated lymphoid tissue (NALT)[51]. Mucosal surfaces are prone to infection as they are thin permeable layers designed for their specific purpose. For example, the gut is designed for food absorption, the lungs for gas exchange and the eyes and nose for sensory activities. As such these sites are vulnerable to Infection and are commonly the route for infectious agents to enter the body[51].

1.11.1 Nasopharynx-associated lymphoid tissue (NALT)

Human nasopharynx-associated lymphoid tissue (NALT) includes tonsils and adenoids. The tonsils and adenoids are part of a ring of lymphoid tissues known as Waldeyer's ring in the nasopharynx[158]. This is an important and unique inductive site for B cell responses. Both tonsils and adenoids are large clusters of mucosal lymphoid tissue which are an important site of immune induction. They are ideally located to be exposed to both airborne and alimentary antigen[159]. During childhood they can often become enlarged due to hypertrophy or infections which can lead to their removal to relieve airway blockage. Tonsils and adenoids contain four specialized lymphoid compartments participating in the immune functions of these organs, namely the reticular crypt epithelium, the extrafollicular area, the mantle zones of lymphoid follicles and the follicular germinal centres (GCs) [160]. Tonsils and adenoids resemble lymph nodes but have no afferent lymph. Instead antigen is transported to T and B cell areas by antigen presenting cells such as dendritic cells[160]. Dendritic cells are found in abundance in extrafollicular areas and are often surrounded by T cells, particularly CD4+ T cells. Various types of these cells including naïve (CD45RA+), memory (CD45RO+) and recently activated (CD25+) are found and can produce both a primary and secondary immune response[159].

Adenotonsillar tissue differs from peripheral blood in that B cells are the predominant lymphocyte population found here. The main T cell population found in adenotonsillar tissue is CD4+ T cells with a low percentage of CD8+ T cells[161].

1.11.2 Consequences of adenotonsillectomy

During this study adenotonsillar tissue was collected from patients who underwent routine adenotonsillectomy. Reasons for surgery were mainly recurrent infections or sleep disorders[162 163]. It is still questionable as to what the effects of removing tonsils are, particularly with regards to the mucosal immune response in the upper respiratory tract. Given that one of the main reasons for surgical removal of adenotonsillar tissue is due to

recurrent infections it is surprising that there have been reports of increased risk of nasopharyngeal colonisation by respiratory pathogens after adenotonsillectomy[162]. It has been shown that removal of tonsils has only a very small effect on reduction of symptoms associated with upper respiratory tract infections, with sore throats and the associated absence from school being reduced marginally[163].

It has also been shown that adenotonsillectomy seems to increase the levels of pneumococcal carriage, particularly when the surgery occurs at a very young age. The pneumococcal polysaccharide specific antibody response is also reduced which certainly indicated that adenotonsillar tissue is a crucial site involved in immune responses against the pneumococcus in children[162].

1.13 Pneumococcal Vaccines

1.13.1 History

The first successful case of pneumococcal vaccination occurred over a hundred years ago in 1911 when British physician Sir Almroth E. Wright and colleagues vaccinated several thousand gold mine workers in South Africa with a crude whole-cell heat-killed pneumococcal vaccine[164]. This vaccine was developed from circulating strains of the bacteria with no regard at all for serotype specificity as at this time it was unknown that the pneumococcus had so many serotypes. Between 1923 and 1929 Avery and Heidelberger conducted work which showed that the polysaccharide capsule of the bacteria was the basis of the different pneumococcal serotypes and that this capsule could be important for the immunogenicity of the bacteria. In 1930 Thomas Francis Jr and William S Tillett injected pure pneumococcal polysaccharide into individuals recovering from pneumococcal pneumonia and discovered that the polysaccharide injected was able to induce specific antibody against the serotype used[165]. Developments in polysaccharide technology and the outbreak of World War II led to the development of a quadrivalent (serotypes 1, 2, 5 and 7) pneumococcal vaccine at a US Arm Air Force technical school in South Dakota[10]. In 1947 E.R

Squibb & Sons obtained a License in the USA to distribute two different formulations of a hexavalent pneumococcal polysaccharide vaccine containing different polysaccharides. One was aimed at adults and contained polysaccharides for serotypes 1, 2, 3, 5, 7 and 8. The other was for use in children and contained polysaccharide for serotypes 1, 4, 6, 14, 18 and 19. At around the same time as pneumococcal polysaccharide vaccine developments, antibiotics were being introduced. Post war doctors preferred to use antibiotics to treat pneumococcal infection and as such interest in the development and use of vaccines faded. Little more would be achieved in the field of pneumococcal vaccine development until the 1970's[10].

1.13.2 Pneumococcal polysaccharide vaccine (PPSV)

Antibiotic therapy as a method of treating pneumococcal disease was shown to be inadequate as it still maintained a high mortality rate. This along with the emergence of antibiotic resistant strains of the pneumococcus led to a resurgence in interest for the development of a pneumococcal polysaccharide vaccine[166]. A 14 valent pneumococcal polysaccharide vaccine was approved in 1977, containing 50 µg of purified polysaccharide capsule from serotypes 1,2, 3, 4, 6, 8, 9, 12, 14, 19, 23, 25, 51 and 56[167]. This vaccine was recommended for use in children 2 years or older who had other chronic health problems and therefore had an increased risk of being infected by the pneumococcus and for people older than 50. This vaccine contained serotypes that were effective against a proportion of invasive pneumococcal diseases but not all. It was recognised that a vaccine targeting a wider range of serotypes was needed which led to the development of a 23 valent vaccine (PPSV23) which was introduced in 1983 and contained 25 µg of capsular polysaccharide from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F[11, 168]. PPSV23 has very good efficacy in immunocompetent adults and older children and is effective against most invasive pneumococcal serotypes[169].

Serotype selection for inclusion in the vaccine was based on their prevalence worldwide. This differs from region to region with some serotypes being present in certain geographical regions. In developed countries PPSV23 provides coverage against up to 90% of invasive pneumococcal infections[164]. In Britain for example, severe pneumococcal infections are between 80-90% likely to be caused by serotypes covered by the vaccine[170]. However, this isn't the case in certain developing countries where protection offered by PPSV23 isn't as effective. A study in Malaysia showed that 71% of invasive pneumococcal diseases are caused by serotypes contained in the vaccine which leaves 29% of invasive infections being caused by serotypes that aren't protected against[171]. This highlights the limitations of this vaccine in its restriction of only protecting against certain serotypes.

1.13.3 Pneumococcal conjugate vaccine (PCV)

Despite the success of pneumococcal polysaccharide vaccines, it soon became clear they had several short comings. The biggest problem is that they are very poorly immunogenic in children under 2 years of age and this age group is one of the most vulnerable to pneumococcal infection[172]. This was a problem that clearly needed to be overcome, and a solution was found in the form of a protein conjugate vaccine in which pneumococcal polysaccharide is conjugated to a protein to improve its immunogenicity. The process of using a conjugate protein in a vaccine wasn't new. It had already been used in haemophilus vaccine with the first protein conjugated vaccines to be licensed in the USA being for *Haemophilus influenza* type b (Hib)[173].

Several different protein candidates were trialled for use in a pneumococcal protein conjugate vaccine. At first, a tetra-valent conjugate vaccine was prepared with outer membrane protein complex of *Neisseria meningitides;* which showed good immunogenicity in 31 toddlers as well as 62 infants in Finland[174]. A subsequent tetravalent vaccine conjugated with diphtheria toxoid was also found to induce a good memory response[175].

Finally, a penta-valent (6B,14,18,19F,23F)[176] and a hepta-valent (4,6B,9V,14,18C,19F and 23F) polysaccharide vaccine conjugated to a nontoxic form of diphtheria toxin CRM_{197} were found to induce a good antibody response in infant and toddlers[177]. A large-scale safety and efficacy trial of the hepta-valent vaccine (PCV7) conjugated to CRM_{197} was carried out in Northern California, USA; which included 37,868 children in a randomly selected double blinded trial[178]. The vaccine was a success with both safety and efficacy being reported as excellent. It was shown that if 3 doses of the vaccine were given at 2-month intervals starting at 2 months of age followed by a booster dose at age 2 the vaccine would effectively protect children from pneumococcal disease[178]. Based on this success PCV7 was licensed in 2000 and is found on the routine immunisation schedule of children in the USA where it is administered in a 3-dose schedule followed by a booster between the ages of 12-15 months[179]. Other studies led to the inclusion of PCV7 on to the vaccination schedule of children in European countries and by 2007 more than 70 countries were licensed to use it[180]. Its use in the USA has seen the rates of invasive pneumococcal disease fall from 97 to 24 cases per 100,000. The vaccine also reduced the incidence of pneumococcal disease seen in unimmunised individuals, perhaps as a direct result of herd immunisation[29].

Despite the success of PCV7 there have been some issues. The use of this vaccine has been associated with serotype replacement in which serotypes contained in the vaccine decrease in incidence but are replaced by those not found in the vaccine[181 182]. The most important example of this is serotype 19A which was not in the vaccine as it was the ninth leading cause of invasive pneumococcal disease in the USA. After PCV7 was introduced it became the number one leading cause of invasive pneumococcal disease in the USA[28]. Serotype 19A also became the leading cause of invasive pneumococcal disease after PCV7 vaccination in Australia, Canada and many European countries[33, 183, 184]. Serotype replacement highlights the need to develop a broad ranging vaccine that is affective against all pneumococcal serotypes as opposed to just a select few[12]. Currently 10 valent (1, 4, 5,

6B, 7F, 9V, 14, 18C, 19F and 23F) and 13 valent (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) pneumococcal conjugate vaccines are used[185]. Children and infants are routinely vaccinated with pneumococcal conjugate vaccines and this has led to a decrease in invasive pneumococcal diseases caused by serotypes contained within the vaccine[186]. There has also been a herd effect of vaccination which has seen a decrease in invasive pneumococcal disease in older children and adults[187 188, 189].This is down to reduced transmission of bacteria within the community due to decreased levels of pneumococcal carriage.

1.13.4 Immune mechanisms of PPSV and PCV

The immune mechanisms of PPSV and PCV differ. PPSV works by inducing antibodies specific to the polysaccharide contained in the vaccine, which induce phagocytosis through activation of compliment and opsonisation of bacteria[168]. B cell receptors on B cells recognise repeating epitopes found in pneumococcal capsular polysaccharide which activates the B cell to convert to plasma cells which secrete antibody specific to the polysaccharide. This process is T cell independent as it occurs without any T cell intervention.[190].

PCV also stimulate B cell receptors in a way that is identical to that of PPSV, but they also have an additional mechanism of action. The vaccine is composed of capsular polysaccharide conjugated to a protein carrier. The capsular polysaccharide activated the B cell response while the protein carrier causes an induction of T cell responses. The protein is processed by antigen presenting cells (APC) and is then presented to CD4+ T helper cells. The T cell response enhances the primary antibody response and provide a stronger secondary response through the induction of both memory B and T cells[191]. The immune mechanisms of PCV are much stronger than those of PPSV and are much more effective in infants and the elderly. Its ability to induce memory B and T cells is also a huge advantage

which provides enhancement of the immune response if a subsequent infection occurs or upon receiving a booster dose of the vaccine[191].

1.13.5 Advantages and disadvantages of PPSV and PCV

PPSV23 was successful in controlling pneumococcal disease in adults but was limited by its poor immunogenicity in young children. New born children are one of the most at risk groups for pneumococcal disease, but they have B cells which respond very poorly to the polysaccharide antigens contained within the vaccine[192]. Despite maturation of the immune system over the first two years of life, the protective effects of this vaccine remain poor. On top of this no memory response is induced so re-vaccination of children does not cause an enhancement of the immune response[193]. To effectively protect against pneumococcal disease using this vaccine, it is necessary to re vaccinate periodically due to the lack of memory response. However, there is evidence that immune tolerance is developed upon repeated exposure[194]. There is also no protection offered against mucosal infection which means it offers little protection against nasopharyngeal carriage of the bacteria and thus does not protect against the spread of the bacteria or against its progression from non-invasive to invasive infection.

Pneumococcal conjugate vaccines offer better protection against pneumococcal disease than polysaccharide vaccines. They provide a much stronger immune response and are effective in infants, young children, elderly people and those who are immunodeficient. It also induces mucosal immunity which confers protection against pneumococcal carriage[195]. This ability to inhibit nasopharyngeal colonisation is a key advantage of the conjugate vaccine over the polysaccharide vaccine as protection against nasopharyngeal colonisation limits the bacteria's progression to an invasive infection and reduces the chance of transmission from person to person. In this way the pneumococcal conjugate vaccine offers herd immunity within a community[196, 197].

A disadvantage of the conjugate vaccine is its high price. This makes them unrealistic for global use. The countries in which pneumococcal diseases burden is highest cannot afford the expense of including PCV13 as part of their vaccination programmes. PCV13 also includes fewer serotypes than PPSV23. This means fewer serotypes are protected against many of which are responsible for invasive pneumococcal disease in at-risk groups of individuals. However, despite its disadvantages, PCV13 is still the best available option for protection against pneumococcal disease, particularly in young children.

1.13.6 Development of protein-based vaccines

Why there is a need to develop a protein based pneumococcal vaccine? Over 90 serotypes of *S. pneumoniae* exist and there is huge variation between which serotypes affect different regions[20]. Current pneumococcal conjugate vaccines are very effective at conferring protection against serotypes included in the vaccine, but they are very complex and incredibly expensive to make. In addition, they do not provide protection against serotypes not included in the vaccine. In contrast to this protein pneumococcal vaccines have the potential to provide protection against all serotypes which would prevent other serotypes replacing ones that are protected against.

For a protein-based vaccine to be effective against pneumococcus, ideally it should be able to induce specific antibody and CD4+ T cell including IL17A dependent immune responses. Antibodies against pneumococcal proteins are important for clearance of bacteria through the promotion of opsonophagocytosis and recent evidence shows that CD4+ T cell responses have an important role to play in protection against pneumococcal infection[47]. CD4+ T cell responses have been implicated in pneumococcal carriage. A protein based pneumococcal vaccine could potentially be used alone or in combination with current capsule polysaccharide-based vaccines to broaden their current protection.

A stand-alone protein vaccine has the potential to be both cheaper and less complex to produce than current pneumococcal conjugate vaccines. However, it would need to be able to demonstrate its effectiveness at achieving the same level of success as a PCV. Of course, its effects would be broader in range which could potentially outweigh any slight reduction in efficacy. Alternatively, one or more pneumococcal proteins could be added to a PCV which could lead to a broadening of coverage and minimise new serotypes emerging to replace ones included in the vaccine. It would have to be tested to ensure the addition of the proteins did confer a greater benefit than just the PCV alone.

1.13.6.1 Current research into pneumococcal protein-based vaccines

Widespread use of 7 valent PCV led to emergence of disease caused by non-vaccine serotypes[28]. New formulations of the vaccine, containing 10 and 13 serotypes have recently been licensed but the same issue of serotype replacement is likely to occur. The cost of producing conjugate vaccines is high, making them difficult to implement worldwide as developing countries cannot afford to use them. With the risk of serotype replacement still likely to occur, vaccine research has moved to looking at protein candidates that would be protective against all serotypes of *S. pneumoniae* as opposed to just a few. A protein pneumococcal vaccine could provide protection against all serotypes of *S. pneumoniae* in a single formulation which would have numerous benefits including reduced manufacturing costs and elimination of the issue of serotype replacement. A cheaper vaccine would be more accessible to developing countries, whose populations are more at risk from pneumococcal infection anyway.

The next generation research for pneumococcal vaccine is focussed on the creation of a vaccine containing several conserved proteins that can induce broader immunity to protect against infection by *S. pneumoniae*. Over 20 pneumococcal proteins have been identified

which produce protective antigens against the pneumococcus including pneumococcal surface protein A (PspA) and pneumolysin (Ply) [198].

Pneumolysin is a highly conserved protein which causes the formation of pores in cell membranes containing cholesterol. Ply is in the cytoplasm of *S. pneumoniae* but is released by autolysis[199]. Ply is a major virulence factor, exerting its effect on different cell types including immune cells and epithelial cells. Antibodies against Ply can be found in humans who have been colonised by *S. pneumoniae* and these antibodies have been shown to passively protect mice that have been challenged with pneumococci[200]. Pneumolysin in its purified form is highly toxic and as such wouldn't be suitable for use in a vaccine targeted to humans. However, Ply can be detoxified which involves its genetic or chemical modification to remove its haemolytic activity[201]. Recently a detoxified genetic mutant of Ply (PlyD1) has been developed which has been used in mouse models and has shown some protection is conferred to the mice involved[202].

Ply has been studied in mouse models and in natural infections in humans. It has been shown to induce both adaptive and innate immune responses and antibodies against Pneumolysin have been found in individuals infected with *S. pneumoniae*[200, 203]. There is limited genetic variation of Pneumolysin between different serotypes of *S. pneumoniae* which suggests it may be a suitable target for inclusion in a vaccine[204].

1.14 TGF-β

The Transforming Growth Factor Beta (TGF-β) superfamily consists of many different proteins including the TGF-beta proteins, Bone Morphogenetic Proteins (BMPs), Growth Differentiation Factors (GDFs), Activins and Inhibins[205]. Transforming growth factor beta (TGF-β) is a multifunctional cytokine that is expressed in 3 different isoforms TGF-β 1-3[206]. TGF-β is secreted by many different cell types has several roles including a role in regulating proliferation, differentiation, adhesion and migration[207]. TGF-β molecules are proposed to

act as cellular switches that regulate processes such as immune function. Many cell types have TGF beta receptors and as such can be either positively or negatively regulated by TGF-β[207]. TGF-β exists in an inactive or latent form and must be activated to be able to exert its suppressive effect[205].

Secreted TGF-β is cleaved into a latency associated peptide (LAP) and a mature TGF-β 1 peptide. In its latent state TGF-β is composed of TGF-β 1 homodimer, a LAP homodimer and a TGF-β 1 binding protein. In its active form is composed of TGF-β 1 homodimer[205].

1.14.1 Latent TGF-β

TGF-β 1, 2 and 3 are all synthesized intracellularly as precursor molecules. After synthesis the TGF-β molecules are secreted as part of an inactive complex comprising of an N-terminal latency-associated peptide (LAP) and a mature TGF-β monomer at the C terminal. Homodimers of TGF-β and LAP interact in a non-covalent manner forming a complex known as small latent complex (SLC)[208]. LAP is then covalently linked to one of three latent TGF-β binding proteins (LGBTs) to form a larger complex called a large latent complex (LLC) which is then secreted out of the cell into the extracellular matrix[209].

The LLC will then remain in the extracellular matrix in an inactive state and requires further processing to release the active TGF-β product[210]. The presence of TGF-β in an inactive complex allow for the closer regulation of TGF-β signalling, as many cells express TGF-β receptors, but it is not always beneficial for TGF-β to be exerting an effect. TGF-β present in its latent state allows it to quickly be cleaved to its active state under the right conditions. The ubiquitous expression of TGF-β and its receptor suggest that its regulation must be complex and multifactorial but one of the most important mechanisms of preventing the effect of TGF-β is by stopping the release of the TGF-β molecule.

1.14.1.1 Enzyme mediated TGF-β activation:

For TGF-β to become activated, the TGF-β molecule must be cleaved form the LAP and LTBP in the LLC. Within a lab, TGF-β can be activated within a sample by lowering the pH to 2.0 for a short period of time. This method will activate all the TGF-β present in a sample[211]. There are other in lab methods which can be used to activate TGF-β but transient acidification is the most effective[212].

TGF-β can be activated by surface receptors and protease activity. Proteases are able to release the LLC from the tissue matrix[213, 214]. When the LLC is free of the matrix, the LAP can bind to surface receptors and undergo a conformational change or be further acted upon by proteases to release TGF-β[212].

1.14.1.2 Receptor mediated TGF-β activation:

Receptors for LAP as part of the LLC have recently been characterised which allow cells to hold latent TGF-β on their surface which can then be activated and delivered to its own or other cells TGF-β receptors[215, 216]. An example of this is seen with macrophages which bind latent TGF-β which has either been produced by the macrophage itself or has come from the environment. The macrophage then activates the latent TGF-β which allows it to bind to its TGF-β receptor[217]. Regulatory T cell activity through TGF-β is thought to involve the binding of latent TGF-β to one cell, which is then activated and delivered to other cells, allowing the suppression of other T cells[218-220].

Several surface binding proteins for LAP have been identified including multiple αV containing integrins. These receptors are found on the surface of many different cell types involved in the repair of wounds and inflammation including dendritic cells, endothelial cells and myofibroblasts[212].

αvβ6 was the first alpha containing integrin to be implicated in TGF-β activation. LAPs contain an RGD motif which is recognized by the majority of alpha containing integrins. αvβ6 activates TGF beta 1 by binding to the RGD motif present in LAP β1 and LAP β3. When bound TGF-β is liberated from its latent complex[221].

In mouse models where the integrin αVβ8 is knocked out on their dendritic cells, autoimmunity and colitis are seen. These dendritic cells become unable to induce the activation of Treg cells, an effect which is reversed if active TGF-β is added[222, 223].

1.14.2 Signalling pathways

TGF-β signalling occurs through a heterotetrameric receptor complex which comprises of a type 1 receptor dimer and a type 2 receptor dimer. The receptors within the complex are serine/threonine kinase receptors with a cysteine rich extracellular domain, a transmembrane domain and a serine/threonine rich domain. Signalling begins with the binding of TGF-β ligand to a TGF-β type 2 receptor[224]. This binding leads to the rotation of the receptors to allow the exposure of the cytoplasmic kinase domains. The type 2 receptor then catalyses the phosphorylation of the serine residues of the type 1 receptor[225]. The type 2 receptor bound depends on the TGF-β ligand as there are different type 2 receptors for each class of ligand.

1.14.3 Smad pathway

Smads are a class of intracellular signalling proteins that mediate the signalling of members of the TGF-β superfamily. Smads can be classified into three distinct groups based on their roles in TGF-β signal transduction. These groups are receptor regulated Smads (R-Smads), common partner Smads (Co-Smads) and inhibitory Smads (I-Smads)[225]. The phosphorylation of the type 1 receptor leads to the recruitment of R-Smads. In most cell types Smad 2 and Smad 3 are recruited and phosphorylated. In other cells Smad 1 and

Smad 5 can be activated depending on which type 1 receptor is expressed by the cell[225]. When the R-Smad protein has been activated it associated with the Co-Smad, Smad 4[226]. This R-Smad/Co-Smad complex is then translocated into the nucleus of the cell where it is responsible for the regulation of gene expression. I-Smads prevent R-Smads from interacting with the TGF-β receptor or compete with Co-Smads to prevent the generation of the R-Smad/Co-Smad complex. I-Smads play a key role in the regulation of TGF-β signalling by negative feedback[227].

1.14.4 TGF-β and regulatory T cells

TGF-β and Treg cells are both important in the control of immune responses and in the maintenance of immune homeostasis. TGF-β appears to play a role as an effector cytokine involved in the immunosuppressive function on Treg cells.

TGF-β 1 has a role in the induction of both inducible Tregs which have an inhibitory role and Th17 cells which are responsible for the production of numerous pro-inflammatory cytokines including IL17A[228].

There are several mouse models that have been established which allow the investigation of the role of TGF-β in immune regulation. TGF-β -/- mice allowed the realisation of how important TGF-β 1is in immune system regulation. The mice in this model develop a rapid wasting syndrome which leads rapidly to death[229]. Transgenic mice that have an impaired TGF-β signalling pathway show an increased susceptibility to the induction of disease, although it is not as severe as disease seen in TGF-β -/- mice[230]. Mice can survive for several months without developing severe disease. This can be explained by TGF-β acting on multiple cell types. So TGF-β can still influence some cells but not others. So, mice show a susceptibility to the induction of allergic and autoimmune responses without developing spontaneous autoimmune disease.

1.15 Integrins

Integrins are transmembrane receptors that act as a bridge for interactions between two cells or between cells and the extracellular matrix[231]. Integrins are responsible for triggering chemical pathways which pass information about the exterior of the cell into the interior of the cell. In this way the chemical composition or the mechanical nature of the cell can be monitored, and events triggered at the cell surface can be rapidly responded to[231]. At least 18 alpha and 8 beta subunits have been identified in humans, which combine to form 24 different integrins.

Integrins possess no enzymatic activity which makes them different to other signalling receptors. Their signalling is reliant on cross talk with other signalling pathways, in particular, growth factor (GF) signalling pathways[232].

The two main functions of integrins are in the attachment of the cell to the extracellular matrix and in signal transduction from the extracellular matrix to the cell[233]. They do play a role in various other biological activities including cell migration and immune patrolling[234].The integrin αvβ8 has been shown to have a role to play in the activation of TG-β 1[235]. In this thesis we have explored the role of the integrin αvβ8 in the activation of TG-β and therefore in the activation of Foxp3+ Treg cells.

1.15.1 TGF-β and the integrin αvβ8 in the development of Treg cells

TGF-β plays a fundamental role in the development of Treg cells and has a role in the induction of both induced and natural Tregs[236]. In addition to roles in Treg cell development, TGF-β plays an important role in the functional ability of Treg cells to suppress T cell responses. As already discussed, TGF-β exists in an inactive state that must be activated in order for it to bind to its receptor and exert a suppressive effect. TGF-β activators include a variety of proteases and cell surface molecules which are able to alter

latent TGF-β to enable the active state to bind to its receptor[237]. Foxp3+ Treg cells have been shown to be able to activate TGF-β via the expression of the integrin αvβ8 and on activated Treg cells, this integrin is upregulated[235]. If Treg cells lack the expression of αvβ8 they become completely unable to suppress T cell mediated inflammatory responses[235].

1.16 Aims/Objectives of this Thesis

The aims of this thesis are as follows

1. To investigate the frequency of Foxp3+ Treg cells in NALT and PBMC in both children and adults.
2. To investigate the frequency of Tr1 cells in NALT and PBMC samples in children and adults.
3. To investigate whether stimulation using Pneumococcal concentrated culture supernatant (CCS) with and without pneumolysin and capsular polysaccharide can activate Foxp3+ Treg cells.
4. To investigate if the toxoid of pneumolysin W433F and purified capsular polysaccharides T3P and 6B can activate Foxp3+ Treg cells in tonsillar MNC.
5. To investigate whether stimulation using pneumococcal CCS with and without the pneumolysin and capsular polysaccharide can activate Tr1 cells in tonsillar MNC.
6. To investigate if the toxoid of pneumolysin W433F and purified capsular polysaccharides T3P and 6B can activate and induce Tr1 cells in tonsillar MNC.
7. To investigate the role of TGF-β in he activation of Foxp3+ Treg cells and the role of the integrin αVβ8

Chapter 2

Materials and Methods

2.1 Subjects and Samples

2.1.1 Subjects

Samples used in this study were collected from children and adult patients undergoing adenoidectomy and/or tonsillectomy due to upper airway obstruction or tonsillitis, attending Alder Hey Children's Hospital and Royal Liverpool and Broadgreen University Hospitals. Patients who had any known immunodeficiency were excluded from the study. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee) and written informed consent was obtained from each patient or their legal guardians as appropriate.

2.1.2 Patients Samples

2.1.2.1 Adenotonsillar Tissues

Immediately after surgery, adenotonsillar tissue was transferred into a 25ml universal tube, containing 10-15ml of Hank's Balanced Salt Solution (HBSS) (Sigma Aldrich, UK) supplemented with 10µg/ml gentamycin (Sigma) and 1% L-glutamine (Sigma Aldrich). The tissue was then transported to the laboratory ready for processing.

2.1.2.2 Peripheral Blood

25 ml universal tubes containing heparin (LEO Pharma, UK), an anticoagulant, were used to collect 2-5 ml of blood prior to surgery. This was then transported to the laboratory, ready for processing on arrival.

2.1.2.3 Nasopharyngeal Swabs

Nasopharyngeal swabs were collected into a sterile vial containing 1ml Skim milk-Tryptone-Glucose-Glycerine (STGG) broth. Before the operation (while patient was anaesthetised), the nasopharyngeal swab sample was collected using a sterile Dryswab™

(Medical Wire & Equipment, UK). The swab was then inoculated into a vial containing STGG, before transportation to the laboratory, where it was stored in a -80°C freezer ready for analysis at a later date.

2.1.2.4 Saliva

Before surgery, a sample of saliva was collected with a sterile 'Oracol' sponge swab (Malvern Medical Developments Limited, UK). The absorbent sponge swab was placed in the mouth and left for approximately one minute to allow absorbance of saliva. Once saturated, the saliva swab was placed into a test tube and transported to the laboratory. Upon arrival at the laboratory the saliva swab was placed in a 20 ml syringe and the saliva was forced out of the swab into a 1.5 ml eppendorf tube. This was then immediately stored in a -80°C freezer ready for analysis later.

2.2 Processing of Samples

2.2.1 Isolation of mononuclear cells from adenotonsillar tissue

All processing of adenotonsillar tissue samples occurred within a class II safety cabinet to prevent any contamination of cells. Adenotonsillar tissue was washed with 5 ml of HBSS two to three times to ensure the sample was clean and ready for processing. 15-20 ml of HBSS was added into a sterile petri dish and the adenotonsillar tissue was transferred into the dish. Using fresh sterilised scalpel and forceps the tissue was mashed for several minutes to release the cells into the media. The media containing the cells was carefully passed through a 70 µm filter (BD biosciences, USA) into a fresh universal tube. In some instances, where a blockage of the filter occurred, several filters were used. Cells were then layered on top of 15-20 ml Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, UK) using a Pasteur pipette. This was done very slowly with the universal held at a slight angle to prevent the filtrate mixing with the ficoll. The sample was centrifuged at 400 x g for 30

minutes with the brake set to 1. After centrifugation a clear interface layer of cells was formed which was then transferred using a Pasteur pipette into a fresh universal tube. This was topped up to 45 ml with sterile PBS solution (Sigma, UK) before being centrifuged at 400 x g for 10 minutes. The supernatant was then poured off into a waste bottle and the pellet was re-suspended using a vortex. Cells were re-suspended in 3-5 ml of sterile RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich) and counted using an automatic cell counter. (BioRad, UK). After counting, each cell suspension was adjusted to contain $4x10^6$ cells/ml concentrations.

2.2.2 Collection of blood serum and isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples were centrifuged at 400 x g for 10 minutes to allow collection of serum. Serum was collected in approximately 500 µl aliquots in 1.5 ml eppendorf tubes and stored at -80°C. Following serum collection, the PBMC's were isolated. To do this, Peripheral blood was carefully layered onto 10 ml Ficoll Paque™ PREMIUM (GE Healthcare Life Sciences, UK). Gradient centrifugation of the sample at 400 x g for 30 minutes allowed the collection of the mononuclear cell layer which was then washed with sterile PBS (Sigma) at 400 x g for 10 minutes. The cells were then re-suspended in 1 ml RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich); and counted in an automated cell counter (BioRad). Cells were then further re suspended in culture medium and the concentration adjusted to 4×10^6 cells/ml.

2.3 Pneumococcal concentrated culture supernatant (CCS)

The strain used in this study was *Streptococcus pneumoniae* encapsulated type 2 strain D39 (NCTC7466)[238]. An isogenic pneumolysin deficient mutant ($Ply^{-/-}$) was also used[239]. Concentrated pneumococcal culture supernatant (Pneumococcal CCS) was prepared from these strains following a method outlined previously[240]. Optimal dose concentrations were determined prior to cell stimulation to be 1 µg/ml.

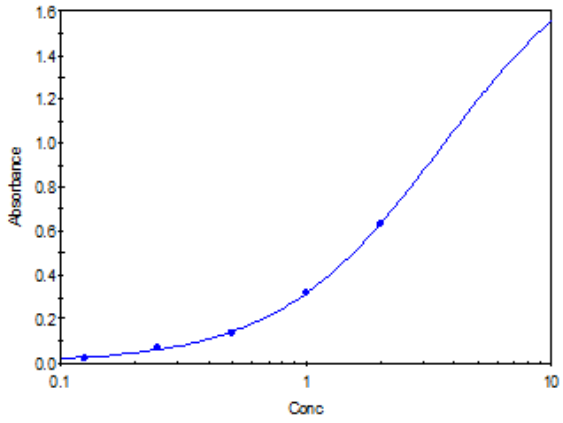
2.3.1 Preparation of pneumococcal CCS

Bacterial frozen stocks were cultured overnight on blood agar plates (Fisher Scientific, UK) (37°C, 5% CO2). After about 18 hours, typical α-haemolytic colonies of *S. pneumoniae* were observed. Several colonies were then used to inoculate Todd Hewitt Broth (THB) (Oxoid, UK) containing 5% yeast extract which was then cultured overnight (37°C, 5% CO2). The following day, the optical density of the culture was measured at 620nm and then this was checked every 30 minutes until exponential phase of growth was reached (OD of 0.4-0.5 at 620nm). The broth culture was centrifuged at 3000 x g for 30 minutes and the supernatant removed. The supernatant was then passed through a 0.45 µm filter followed by a 0.2 µm filter. Concentration of the pneumococcal culture supernatant was then achieved by centrifuging the sample at 3000 x g for 30 minutes in a Vivaspin15 concentrator (Sartorius Stedim Biotech, Germany). This process was repeated several times to ensure that the sample was concentrated tenfold. Samples were then aliquoted into 1.5 µl microcentrifuge tubes and stored at -80°C until use.

2.3.2 Measurement of protein concentration in pneumococcal CCS.

The protein concentration of pneumococcal CCS was measured using the Bradford protein assay and involved the use of Bradford protein dye reagent (Sigma) following manufacturer's instructions. The principle of this assay is to compare sample dilutions

against a known standard curve in order to work out the concentration of your sample. The standard curve used is a Bovine Serum Albumin (BSA) standard curve. A stock concentration of 2 mg/ml of BSA was serially diluted (two-fold) in sterile PBS five times to give the BSA curve. Samples were diluted serially (ten-fold) three times. 5 µl of sample or BSA standard was added to the appropriate well of a 96-well Costar plate and 250 µl of Bradford reagent was then added. The plate was incubated in the dark for 5 minutes before absorbance was read at 595nm using the ThermoElectron MultiSkan (Opsys MR, Thermo labsystems, UK). A standard curve was produced from the readings of the BSA dilutions and this was used to work out the concentrations of the samples using DeltasoftPC microplate reader software. (Biometallics Inc., USA).



BSA Standard	Concentration mg/ml	Mean OD
1	0.125	0.021
2	0.25	0.070
3	0.5	0.141
4	1	0.317
5	2	0.633

Figure 2.3.1: Standard curve of Bradford protein assay

Pneumococcal CCS	Concentration (mg/ml)	Concentration used to simulate cells
D39 WT	0.641	1µg/ml
Ply-/-	0.733	1µg/ml

Table 2.3.1: Concentration of Pneumococcal CCS measured by Bradford protein assay

2.4 Western Blotting

2.4.1 Principle of the assay

This method is a widely accepted analytical technique in which proteins are separated on sodium dodecyl phosphate- polyacrylamide gels (SDS-PAGE), according to their molecular weight. These proteins are then transferred into a nitrocellulose or PVDF membrane. Non-specific binding is blocked with bovine milk (skimmed) protein, before the target protein is detected using antibodies specific to the target protein. A primary antibody is added which is specific the target protein before the addition of a secondary antibody specific to the primary antibody. The Secondary antibody commonly labelled with horseradish peroxidase or alkaline phosphatase cleaves a chemiluminescent agent to provide luminescence in proportion to the amount of protein present[241].

2.4.2 Western Blotting of Pneumococcal CCS

To confirm the presence of pneumolysin in the WT and absence of Pneumolysin in the Ply-/- mutant strain western blotting was used. This involved the following a multi-step process which is outlined below.

2.4.2.1 Gel Electrophoresis

Samples were prepared by diluting 1:2 using Laemmli reducing Buffer[242] before being heated on a heat block at 100°C for 5 min. The gel was loaded into the tank before the addition of running buffer into the middles and side of the chamber. The comb was gently removed to prevent any tearing of the gel. 20 µl of prepared WT, Ply-/- and Cap -/- pneumococcal CCS and 5 µl of precision plus protein kaleidoscope ladder (Bio-Rad, UK) were loaded into the wells using long sample loading tips. Electrodes were connected between the tank and the power pack and run for 1 hr at a constant 250v in 50mA.

2.4.2.2 Protein Transfer by Western blotting

The gel was removed from the tank after gel electrophoresis and carefully placed in transfer buffer. The stacking gel was gently cut from the resolving gel using a sharp scalpel. The transfer of the protein was performed using a Transblot TurboTM transfer system (BioRad) into a 0.2µm nitrocellulose membrane (Transblot turbo transfer pack). Protein is transferred using electroblotting which involves the use of a current to pull the protein from the gel onto the nitrocellulose membrane. The gel was placed onto a nitrocellulose membrane which was then placed onto the bottom ion reservoir (anode) stack. The top ion reservoir (cathode) stack is then placed on top of the gel and a constant current of 25v in 1000mA was applied for 10 minutes.

2.4.2.3 Detection of Pneumolysin (Ply) proteins in pneumococcal CCS

Following transfer of the protein, the nitrocellulose membrane was blocked with 5% skimmed milk in TBS containing 0.05% Tween20 (TBS-T) for 2 hr at room temperature (RT). The membrane was then washed 3 times in TBS-T before the addition of rabbit anti-Ply (diluted 1:10000 in blocking solution) was added. Next, the membrane was incubated at room temperature for 2 hours before being washed 5 times in TBS-T. The membrane was then incubated with secondary antibody, donkey anti-rabbit IgG-HRP (diluted 1:10000 in blocking solution) for 1 hour at room temperature. Following incubation, the membrane was washed 5 times with TBS-T and then once in PBS before the addition of substrate to the membrane. The substrate was prepared by adding 1:1 volume of chemiluminescence reagent A and B (BioRad). The substrate and membrane were incubated for 5 minutes in the dark at room temperature before the removal of excess substrate at which point the membranes were imaged using the Chemi-DocXRS system (BioRad)

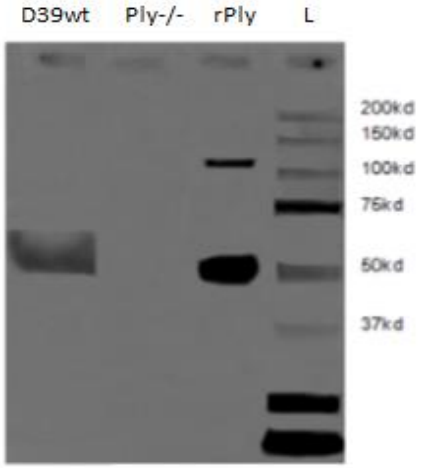


Figure 2.4.2.3 Western blot analysis of pneumococcal CCS Nitrocellulose membrane blotted with pneumococcal CCS were immunostained with rabbit anti-Ply antiserum showing that pneumolysin is present in D39 WT and absent in Ply-/-

2.5 Analysis of immune cells by Flow Cytometry

2.5.1 Principle of test

Flow cytometry is a laser-based technology which allows the measurement and analysis of both physical and chemical characteristics of single cells within a fluid as they pass through a focussed laser beam. Flow cytometry allows the measurement of a cells size, granularity and fluorescence. As the cells within a fluid suspension pass through the interrogation point of the flow cytometer, light is scattered in all directions. Light scattered in the forward direction or forward scatter (FSC) represents the size of the cells with larger cells being represented by a higher forward scatter signal. Light scattered in a sideways direction or side scatter (SSC) represents the granularity of cell. More granular cells are shown to have a higher side scatter signal. Within a sample containing a mixed population of cells, FSC and SSC allow the distribution of different subsets of cells. As well as FSC and SSC, flow cytometry can be used to measure the fluorescence of a cell. Cells which are fluorescently

labelled and are then excited by the laser, emit light of varying wavelengths which allow further characterisation of the cell[243-245].

Flow Cytometric analysis in this project was carried out using the BD FACS Calibre (BD Biosciences). Different subsets of lymphocytes were defined with multiple parameters, including FSC and SSC based gating. Fluorescence emissions of different fluorochromes were measured at different wavelengths; FITC or Alexafluor488 in FL1 at 519nm, PE in FL2 at 578nm, PerCPCy5.5, PECy5 or PECy7 in FL3 at 695nm, and APC or Alexafluor660 in FL4 at 660nm. Data was acquired with Cell Quest software (BDBiosciences) and analysed with WinMDI 2.9 software

2.5.2 Intracellular cytokine staining

2.5.2.1 Principle of intracellular cytokine staining

Cytokine production is an important part of the immune response. Specific cytokines are rapidly produced and secreted upon cellular activation by the correct stimulus under the correct conditions. Cytokines play an important role in many different pathways including the induction and activation of different T cell subsets[246].

Intracellular cytokine staining (ICS) is a very widely used flow cytometry-based assay which is used to detect cytokines within the endoplasmic reticulum after cell stimulation. During ICS, cells are activated using either a specific peptide or a non-specific mix. After activation cells are incubated with a protein transport inhibitor such as Brefeldin A (BFA), by which the accumulated cytokines are retained within the endoplasmic reticulum of the cell. Cells are then washed before being stained with specific antibodies to cell surface markers (e.g. CD3, CD4 or CD8). Finally, cells are fixed, and cell membrane was permeabilised before the addition of antibodies specific to the cytokine that to be detected.

2.5.2.2 Identification of regulatory T cells

To detect the presence of Foxp3+ regulatory T cells and Tr1 cells, cells were stained with antibodies specific to each cell type. Immediately after sample processing, 500 µl of cells from adenotonsillar tissue diluted to $4x10^6$, or 500 µl of cells from peripheral blood diluted to $2x10^6$ were added to wells of a 48-well flat bottom culture plates (Corning Inc., USA). BFA was added and the plates were incubated at 37°C for 4 hours. Intracellular staining of cells using anti-Foxp3 or anti-IL-10 antibodies allowed the identification of these cells for Foxp3+ T regulatory cells or IL10-producing Tr-1 cells in both adenotonsillar tissue and peripheral blood.

2.5.3 Staining procedure

2.5.3.1 Foxp3+ Treg cell staining

Foxp3+ Treg cells have an important role in the prevention of auto immune disease and in the maintenance of immune tolerance. Foxp3+ Treg cells possess several surface markers which can be used to distinguish them from other cell types. These include CD25, CTLA-4, CD103 and CD39. Foxp3+ Treg cells also express the intracellular transcription factor Foxp3 and this is a marker specific to both thymic and peripheral Foxp3+ Treg cells.

To determine the presence of Foxp3+ Treg cells, after the 4-hour incubation with BFA, adenotonsillar MNC were harvested from the plates into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02% PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing, 5 µl of PeCy7 (BD Bioscience) conjugated CD4 was added to each sample and before they were incubated at 4°C for 30 minutes. Cells were then washed using 1 ml of FACS staining buffer, followed by centrifugation at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and they were incubated for 30 minutes at room temperature. Cells were then washed, followed by centrifugation at

600 x g for 8 minutes in permeabilisation buffer before the addition of 15 µl of Alexafluor 647 (BD Bioscience) conjugated Foxp3 to each sample. This was incubated at room temperature for a further 30 minutes, followed by a final wash step using 1 ml of permeabilisation buffer per sample for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

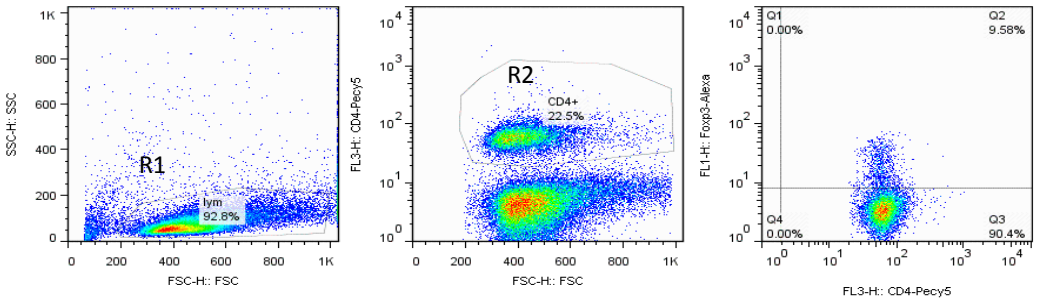


Figure 2.5.3.1. A representative figure which shows the gating strategy used for the identification of Foxp3+ Treg cells (CD4+Foxp3+) in freshly isolated tonsillar MNC.

R1 highlights the lymphocyte population, defined using typical FSC and SSC. Within the lymphocyte population the CD4+ cells are then defined in R2. Finally, the Foxp3+ Treg cells are identified by the gating of the CD4+ population (R2) and the positive staining of Foxp3. This CD4+Fox3+ cell population is defined as the frequency of Treg cells and is shown as a percentage

2.5.3.2 Tr1 cell staining procedure

Tr1 cells are a suppressive subset of regulatory T cell which exert their effect mainly though the production of IL10. Tr1 cells can be identified by the co expression of surface markers CD49b and Lag 3 and the high expression of IL10[1]. This is the combination of markers I was staining for to identify a cell population as being Tr1 cells.

To detect Tr1 cells, after incubation with BFA, adenotonsillar MNC were harvested from the plates into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02% PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing, 20 µl of Alexafluor 647 (BD Bioscience) conjugated CD49b, and 5 µl of PeCy7 (BD Bioscience) conjugated CD4 was

added to each sample and tubes were incubated at 4 degrees for 30 minutes. Adenotonsillar MNC or PBMC were then washed using 1 ml of FACS staining buffer at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and tubes incubated for 30 minutes at room temperature. Cells were then washed in permeabilisation buffer followed by centrifugation at 600 x g for 8 minutes, before the addition of 5 µl of APC (BD Bioscience) Conjugated IL10 and 20 µl of PE (R&D Systems) conjugated LAG3 to each sample. This was incubated at room temperature for a further 30 minutes followed by a final wash step using 1 ml of permeabilisation buffer per sample, followed by centrifugation for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

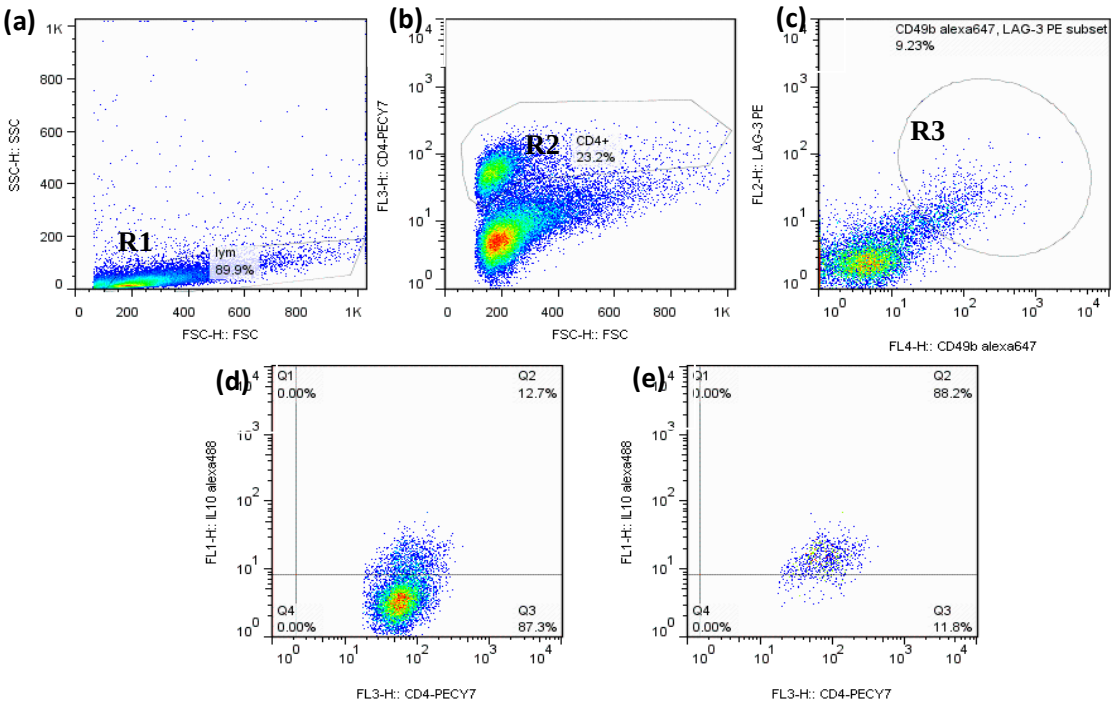


Figure 2.5.3.2. A representative figure which shows the gating strategy used for the identification of Tr1 cell frequency in freshly isolated tonsillar MNC and PBMC.

The lymphocyte population is highlighted in R1, using the typical FSC and SSC configuration. (b) Within the lymphocyte population the CD4+ cells are defined in R2. (c) R3 then highlights the cells within R2 which are both CD49b+ and LAG3+ and are therefore defined as Tr1 cells. (d) IL10 positive cells within the CD4+ lymphocyte population. (e) In order to confirm the cells in R3 are Tr1 cells they are then gated against IL10.

2.5.4 Activation of regulatory T cells

2.5.4.1 Foxp3+ Treg cells

2.5.4.1.2 Cell stimulation

500 µl of tonsillar MNC suspension at a concentration of 4 x 10^6 was stimulated for 3 days and kept in an incubator at 37°C in 5% CO2. After 3 days, 0.5 µl of a protein transport inhibitor, Brefeldin A (BFA) was added to each sample before further incubation at 37°C in 5% CO2 for 4 hours. The incubation was then stopped, the plate wrapped in cellophane and left in the fridge overnight.

2.5.4.1.3 Cell harvest and staining

The following morning cells were harvested from the plated into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02 %PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing 50 µl of a master mix containing 20 µl of CD25 and 5 µl of CD4 were added to each sample and incubated at 4°C for 30 minutes. Cells were then washed using 1 ml of FACS staining buffer at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and incubated for 30 minutes at room temperature. Cells were then washed in permeabilisation buffer followed by centrifugation at 600 x g for 8 minutes before the addition of 50 µl of a master mix containing 15 µl of Foxp3 was added to each sample. This was incubated at room temperature for a further 30 minutes followed by a final was step using 1 ml of permeabilisation buffer per sample for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

2.5.4.2 Activation of Tr1 cells

2.5.4.2.1 Cell stimulation

500 µl of tonsillar MNC suspension at a concentration of 4 x 10^6 was stimulated for 3 days and kept in an incubator at 37°C in 5% CO2. After 3 days 0.5 µl of a protein transport inhibitor, Brefeldin A was added to each sample before further incubation at 37°C in 5% CO2 for 4.5 hours. The incubation was then stopped, the plate wrapped in cellophane and left in the fridge overnight.

2.5.4.2.2 Cell harvest and staining

The following morning cells were harvested from the plate into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02%PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing 50 µl of a master mix containing 20 µl of CD49b, 20 µl of LAG3 and 5 µl of CD4 were added to each sample and incubated at 4 degrees for 30 minutes. Cells were then washed using 1 ml of FACS staining buffer at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and incubated for 30 minutes at room temperature. Cells were then washed at 600 x g for 8 minutes in permeabilisation buffer before the addition of 50 µl of a master mix containing 5 µl of IL10 was added to each sample. This was incubated at room temperature for a further 30 minutes followed by a final was step using 1 ml of permeabilisation buffer per sample for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

2.5.4.3 Induction of Tr1 cells

2.5.4.3.1 Principle of MACS separation

MACS cell separation allows cells which have a certain marker to be isolated from a cell population. This can be either positive selection to obtain a pure cell population or negative selection to remove a cell type from the whole cell population.

The cells which are to be depleted are magnetically labelled with specific microbeads which bind to specific target molecules. In this case, with CD45RO beads and CD25 beads. The cells are then passed through a MACS LD column which is placed within magnetic field of a MACS cell separator. The unlabelled cells which do not express the target molecule run straight through the column and are collected into a tube. This depleted cell population does not contain cells expressing the target molecule. The magnetically labelled cells which express the target molecule are retained on the column.

2.5.4.3.2 Depletion for induction of Tr1 cells

To remove any pre-existing activated Tr1 cells, CD45RO+ cells were first depleted from the cell culture. CD45RO is expressed on several different cell types. It is found on both memory CD4+ and CD8+ T cells as well as being present on CD4+ effector T cells, macrophages and monocytes. The depletion of CD45RO+ cells produces a naïve cell population allowing the analysis of naïve CD4+ T cells by flow cytometry.

2.5.4.3.3 Magnetic labelling and depletion of CD45RO

Approximately 50 x 10^6 cells were taken from the pure cell mix and re-suspended in 400 µl (80µl/107 cells) of depletion buffer (0.5% PBS BSA). To this 100µl (20µl/107 cells) of CD45RO magnetic beads (Miltenyi Biotech) were added. The cell suspension was thoroughly vortexed followed by incubation at 4 degrees for 15 minutes. Following incubation, cells were washed with 10 ml (2 ml/107 cells) of depletion buffer at 400 x g for 10 minutes. The cell pellet formed was re-suspended in 250µl (50µl/10 cells) before magnetic separation was carried out. The MACS LD column (Miltenyi Biotech) was placed inside the magnetic groove of the magnet and primed by the addition of 2 ml of depletion buffer, with acre being taken to avoid bubbles. The cell suspension was then added to the column carefully and allowed to pass through. This process could take some time depending on the number of CD45RO cells present in the sample. After all the cell

suspension had passed through the column, 2 ml of depletion buffer was passed through the column. The collected CD45RO- T cells collected by this process were then re suspended in RPMI medium to a concentration of 4 x 106 cells/ml. Analysis of these cells through stimulation using pneumococcal antigens and FACS analysis was performed following techniques described previously for non-depleted lymphocytes.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA) for measurement of cytokine production in cell culture supernatant

2.6.1 Principle of assay

Enzyme Linked Immunosorbent Assay (ELISA) is a highly sensitive biochemical assay technique designed for detecting and quantifying substances. The basis of the test is to use antibodies and colour change to identify a specific substance. The assay involves the use of a specific monoclonal antibody to coat a microtiter plate. When the sample which is being analysed is added to the plate, the specific antibody that has coated the plate will capture the protein of interest. A second monoclonal antibody is then added to the plate which is specifically designed for detection. The detection antibody is labelled with biotin to allow the subsequent binding of a streptavidin conjugated enzyme. Throughout the addition of each antibody and enzyme, the plate is washed which allows for the removal of any unbound protein. The final step is the addition of substrate which causes a colour change proportional to the amount of protein bound. A standard curve is created which allows the concentration of the specific protein in the sample to be determined[247, 248].

2.6.2 Measurement of IL-10

In vitro production of IL-10 in adenotonsillar MNC cultures was measured using the human IL-10 ELISA Ready-Set-Go® set (eBioscience, UK) following manufacturer's instructions. Capture antibody in coating buffer was used to coat 96-well Costar plates (Corning). The

plates were sealed and left overnight at 4°C. The following morning the plate was washed 5 times with PBS-T using a plate washer. After washing, plates were blotted on absorbent paper to remove any residual buffer. 5 x concentrated assay diluent was diluted to 1 x assay diluent by mixing with 4 parts dH_20. Each well was blocked using 200 µl of 1 x assay diluent. The plate was then incubated for 1 hour at room temperature. During the incubation period the samples and the highest concentration of the standard were prepared. The highest concentration of the standard (500 pg/ml) was prepared by adding 5 µl human IL-10A recombinant protein into 10 ml of 1 x assay diluent. Samples were prepared by diluting 1:20 in 1x assay diluent. When the incubation was over 100 µl of samples and standards were added to the plate. The standards were prepared in duplicate starting from the highest concentration at the top followed by 2-fold serial dilution to give a total of 16 standards. The plate was incubated for 2 hours at room temperature. During the incubation detection antibody was prepared by adding 44µl of purified anti-human IL-10 Biotin to 11 ml of 1 x assay diluent (1:250 dilution) At the end of the incubation period the plate was washed 5 time using PBS-T before the addition of 100 µl of detection antibody to each well. The plate was incubated for 1 hour at room temperature before being washed 5 times with PBS-T. 100 µl of Avidin-HRP (diluted 1:250) was added to each well before incubation for 30 minutes at room temperature. The plate was washed 5 times with PBS-T before the addition of 100 µl of TMB substrate solution to each well. The plate was then incubated in the dark for 15 minutes at room temperature before the addition of 50 µl of stop solution (1M H_2SO_4). The final step was the reading of the plate at 450 nm using a plate reader. The concentration (pg/ml) was calculated using the standard curve generated using DeltasoftPC software (Biometallics).

2.6.3 Measurement of TGF-B

In vitro production of TGF-β in adenotonsillar MNC cultures was measured using the human TGF-β ELISA Ready-Set-Go® set (eBioscience, UK) following manufacturer's instructions. Capture antibody in coating buffer was used to coat 96-well Costar plates (Corning). The plates were sealed and left overnight at 4 °C. The following morning the plate was washed 5 times with PBS-T using a plate washer. After washing, plates were blotted on absorbent paper to remove any residual buffer. 5 x concentrated assay diluent was diluted to 1 x assay diluent by mixing with 4 parts dH_20. Each well was blocked using 200 µl of 1 x assay diluent. The plate was then incubated for 1 hour at room temperature. During the incubation period the samples and the highest concentration of the standard were prepared. The highest concentration of the standard (500pg/ml) was prepared by adding 5 µl human TGF-β recombinant protein into 10 ml of 1 x assay diluent. Samples were prepared by diluting 1:20 in 1 x assay diluent. When the incubation was over 100 µl of samples and standards were added to the plate. The standards were prepared in duplicate starting from the highest concentration at the top followed by 2-fold serial dilution to give a total of 16 standards. The plate was incubated for 2 hours at room temperature. During the incubation detection antibody was prepared by adding 44 µl of purified anti-human TGF-β Biotin to 11 ml of 1 x assay diluent (1:250 dilution) At the end of the incubation period the plate was washed 5 time using PBS-T before the addition of 100 µl of detection antibody to each well. The plate was incubated for 1 hour at room temperature before being washed 5 times with PBS-T. 100 µl of Avidin-HRP (diluted 1:250) was added to each well before incubation for 30 minutes at room temperature. The plate was washed 5 times with PBS-T before the addition of 100 µl of TMB substrate solution to each well. The plate was then incubated in the dark for 15 minutes at room temperature before the addition of 50 µl of stop solution (1M H_2SO_4). The final step was the reading of the plate at 450 nm

with a plate reader. The concentration (pg/ml) was calculated using the standard curve generated using DeltasoftPC software (Biometallics).

2.7 Statistical Analysis

Data were analysed using GraphPad Prism version 5. Initially data was tested for normality using D'Agostino and Pearson omnibus normality test. Analysis of data that did not pass the normality test was then done using non-parametric tests while the data that did pass the normality test was analysed using parametric tests. Differences between two groups, for example adults and children, were analysed by Student's t (parametric) or Mann-Whitney (non-parametric) test. Differences between paired samples for example stimulated tonsillar MNC or stimulated blood samples and unstimulated control cells, were analysed by paired t (parametric) or Wilcoxon matched-pairs signed rank (non-parametric) test.

A p value of <0.05 was taken as a level of statistical significance.

Chapter 3

Characterisation of Foxp3+ T regulatory and Type 1 regulatory T cells in Human Nasal Associated Lymphoid Tissue and Peripheral Blood Mononuclear Cells

3.1 Introduction

Regulatory T cells have been shown to have an important role to play in the modulation of different immune responses during auto immune diseases, tumours, transplantation and allergy[249]. They have also recently emerged as being key players in immune regulation during microbial infection[250]. They are important in the control of excessive effector immune responses which if allowed to continue unchecked, could lead to severe host tissue and cellular inflammation and end up causing more harm than the initial bacterial infection. There are several populations of regulatory T cell including CD8+ regulatory T cells and CD4+ regulatory T cells. Of the CD4+ Treg cells populations that have been identified include Foxp3+ regulatory T cells, Th3 cells and Type 1 regulatory cells (Tr1)[251-253]. (Figure 3.1)

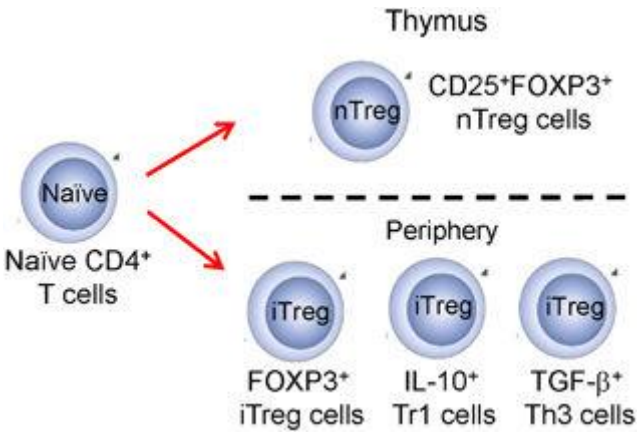


Figure 3.1 Different subsets of Treg cells. The naturally occurring CD4+CD25+FOXP3+ Treg (nTreg) cells are generated in the thymus, whereas three different subsets of inducible Treg (iTreg) cells can be generated in the periphery: (i) FOXP3+Treg cells, (ii) CD4+FOXP3− IL-10–producing Tr1 cells and (iii) TGF-β-expressing TH3 cells. (From: Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β[254])

A naturally occurring suppressive subset of T cells was first described in the 1970's[94]. However due to a series of negative results the hypothesis was rejected. The "suppressor T cell" hypothesis was revived by studies showing that a subset of CD4+ T cells which express CD25 are essential for control of autoreactive T cells in vivo[95]. It was shown that depletion

of CD25+ T cells in adult mice led to the development of various autoimmune diseases including gastritis[95]. This work led to the classification of a naturally occurring CD4+CD25+ subset of Treg cells. Recently, the discovery of a transcription factor which is expressed on this cell type, the forkhead-winged helix transcription factor forkhead box P3 (FoxP3), has allowed this cell type to be distinguished from other CD4+CD25+ T cells[96].

3.1.1 Foxp3+ Treg cells

Foxp3+ Treg cells are the most well studied, and therefore the easiest to categorise. They can be natural (nTreg) or inducible (iTreg). (Figure 3.1) nTregs become mature in the thymus and make up approximately 5-10% of the circulating peripheral CD4+ T cell population[255]. nTregs are produced in the thymus and subsequently migrate to the peripheral blood where they remain, monitoring self-antigen[256]. iTregs acquire their suppressive activity upon their activation by antigen in the periphery and are specific to the antigen, which activates them. nTregs are thought to have an important role to play in maintaining self-tolerance whereas iTregs are thought to be more important at mucosal surfaces where they are more likely to encounter foreign antigen.

Foxp3+ Treg cells possess several surface markers which can be used to distinguish them from other cell types. These include CD25, CTLA-4, CD103 and CD39. Foxp3+ Treg cells also express the intracellular transcription factor Foxp3 and this is a marker specific to both thymic and peripheral Foxp3+ Treg cells.

Foxp3+ Treg cells use the αβTCR for antigen recognition and are restricted by MHC-II molecules as with other CD4+ T cells[101]. It is thought that there are 2 mechanisms by which Treg cells exert their suppressive effects. The contact dependent method involves CTLA-4 on the Treg cell and CD80/86 on the effector T cell[102]. CTLA-4 is expressed at high levels on CD4+CD25+ Treg cells and it is thought that the CTLA-4 interacts with the CD80/86 on the effector T cell to decrease effecter T cell function. Immunosuppressive cytokines are also

important in Treg associated suppression. IL-10 and TGF-β released by Treg cells are responsible for down regulating MHC II and co-stimulatory molecules on dendritic cells which affects their ability to present antigens and so stops the activation of CD4+ T cells[102].

3.1.2 Tr1 cells

Tr1 cells were first described by Roncarolo et al in 1997 who showed a Foxp3- CD4+ T cell subset, which suppressed antigen specific T cell responses, which prevented colitis[105]. Tr1 cells are not as well studied as Foxp3+ Treg cells. They were initially identified based on their cytokine profile. This allows them to be distinguished from other regulatory and effector T cells. They express high levels of IL-10, small amounts of IL4 and IL17 which allow them to be distinguished from Th2 and Th17 cells, and a low level of IL2 which distinguishes them from Th1 cells[98]. More recently it has been shown that Tr1 cells can be identified by the co expression of surface markers CD49b and LAG-3 and the high expression of IL-10[1]. This is the combination of markers I was looking for during this experiment to identify a population as being Tr1 cells.

The original study that led to the discovery of IL-10 producing Tr1 cells began in the late eighties[104]. Tr1 cells were discovered in the peripheral blood of patients with severe combined immunodeficiency (SCID) with long term mixed chimerism after HLA-mismatched fetal liver hematopoietic stem cell transplant (HSCT)[104]. The SCID patients developed long term tolerance to stem cell allografts which suggested there was something regulating immune cells. Isolated cells did not show the same cytokine profile as Th1 or Th2 cells which suggested a unique cell type was responsible for the immune cell regulation. A few years later Groux et al demonstrated that Tr1 cells, producing high amounts of IL10, are a distinct subset of CD4+ T cells that are antigen specific and show strong immunosuppressive activity[105].

Tr1 cells can control the activation of naive and memory T cells and suppress Th1 and Th2 cell mediated immune responses. The suppressive effects of Tr1 cells are reversed by blocking antibodies against IL-10, showing that the inhibitory capacity of Tr1 cells is mainly mediated through production of IL-10. They also kill myeloid cells by the secretion of Granzyme B[98].

3.1.3 Nasopharynx associated lymphoid tissue

The lymphoid tissue found in the nasal mucosa is known as nasopharynx associated lymphoid tissue (NALT) and is part of the mucosal associated lymphoid tissue (MALT)[257]Human NALT includes tonsils and adenoids [258]. Both tonsils and adenoids are large clusters of mucosal lymphoid tissue which are an important site of immune induction. They are ideally located to be exposed to both airborne and ingested antigen[159]. It is common during childhood for them to become enlarged and inflamed due to recurrent infections. This can lead to their removal.

Tonsils and adenoids contain four specialized lymphoid compartments participating in the immune functions of these organs, namely the reticular crypt epithelium, the extrafollicular area, the mantle zones of lymphoid follicles and the follicular germinal centres (GCs) [160]. Tonsils and adenoids resemble lymph nodes but have no afferent lymph. Instead antigen is transported to T and B cell areas by antigen presenting cells such as dendritic cells[160]. Dendritic cells are to be found in abundance in extrafollicular areas and are often surrounded by T cells, particularly CD4+ T cells. Various types of these cells including naïve (CD45RA+), memory (CD45RO+) and recently activated (CD25+) are found and can produce both a primary and secondary immune response[159].

Adenotonsillar tissue differs from peripheral blood in that B cells are the predominant lymphocyte population found here. The main T cell population found in adenotonsillar tissue is CD4+ T cells with a low percentage of CD8+ T cells being found[161].

In this study we aimed to study the numbers of Foxp3+ Treg and Tr1 cells in nasopharynx-associated lymphoid tissue (NALT) and peripheral blood and identify any differences in numbers between the two compartments. We also aimed to study the numbers of these cells and any differences between adult and children samples.

3.2 Aims of the study

In this chapter we have investigated:

1) The frequency of Foxp3+ Treg cells in NALT and PBMC in both children and adults
2) The frequency of Tr1 cells in NALT and PBMC samples in children and adults.

3.3 Experimental Design:

To determine the frequency of Foxp3+ Treg and Tr1 cells within NALT and peripheral blood, freshly isolated MNC from tonsil samples and PBMC samples were stained with fluorescently labelled antibodies to specific cell surface and intracellular markers. For detection of Foxp3+ Treg cells, cells were stained with CD4 and Foxp3. For detection of Tr1 cells, cells were stained with CD4, CD49b, LAG3 and IL10. Cells were then analysed by flow cytometric analysis on a FACSCalibur.

3.3.1 Human subjects and samples

Samples of Tonsillar tissue were obtained from children undergoing routine adenotonsillectomy between the ages of 2-16 years of age. All children involved in the study gave their permission to be involved through informed consent of their parent or guardian.

3.3.2 Detection of Foxp3+ Treg cells

Mononuclear cells (MNCs) were isolated from samples of tonsillar tissue and peripheral blood by ficoll gradient centrifugation. These freshly isolated cells were then stained with

CD4 and incubated in the dark for 20 minutes at room temperature (RT). Cells were centrifuged at 600 x g for 8 minutes before the addition of 350 µl of fixation/permeabilisation buffer. Cells were incubated at 4°C for 30 minutes followed by further centrifugation at 600 x g for 8 minutes. Cells were then incubated with Foxp3 for 30 minutes in the dark at RT. Permeabilisation buffer was then added before centrifugation at 600 x g for 8 minutes. Finally, cells were re suspended ready for FACS analysis on the FACSCalibur. A lymphocyte gate was set based on typical FSC/SSC characteristics and Foxp3+ Treg cells were expressed as the percentage of CD4+ cells which stained positively for Foxp3 (Figure 1). The data gathered from flow cytometric analysis was then analysed using WinMDI.

3.3.3 Detection of Tr1 cells

Freshly isolated MNCs from both tonsillar tissue and peripheral blood samples were stained for the detection of Tr1 cells. Cells were initially stained for surface markers, followed by intracellular markers. For staining of the surface markers, CD4 and CD49b were added to the cells before they were incubated in the dark for 20 minutes at room temperature. Cells were then centrifuged for 8 minutes at 600 x g before the addition of 350 µl of fixation/permeabilisation buffer and incubation at room temperature for 30 minutes. Fluorescently labelled IL-10 and LAG-3 were then added to the cells before incubation at RT in the dark for 30 minutes. Cells were centrifuged before the addition of 1 ml of permeabilisation buffer. Finally, cells were once again centrifuged before being re suspended in FACS buffer ready for FACS analysis on the FACSCalibur. The FACS data gathered was then analysed using WinMDI.

3.3.4 Statistical analysis

Data were analysed using GraphPad Prism version 5. Differences between two groups, for example adults and children, were analysed by Student's t (parametric) or Mann-Whitney

(non-parametric) test. Differences between paired samples, for example stimulated tonsil and stimulated blood samples, were analysed by paired t (parametric) test.

A p value of <0.05 was taken as a level of statistical significance.

3.4 Results

3.4.1 The frequency of Foxp3+ Treg cells in tonsillar MNC and PBMC samples

The frequency of Foxp3+ Treg cells in tonsillar MNC and PBMC was shown by the staining of cells using markers specific for this cell type. A combination stains against surface marker CD4 and the intracellular transcription factor Foxp3 were used. Cells that were CD4+Foxp3+ were concluded to be Foxp3+ Treg cells. A representative figure showing gating strategy can be seen in figure 3.4.1a. Figure 3.4.1b shows that the mean frequency of Foxp3+ Treg cells in children's tonsillar MNC (10.4%) was significantly higher than in PBMCs (4.6%) and that the mean frequency of Foxp3+ Treg cells in adult tonsillar MNC (6.8%) was significantly

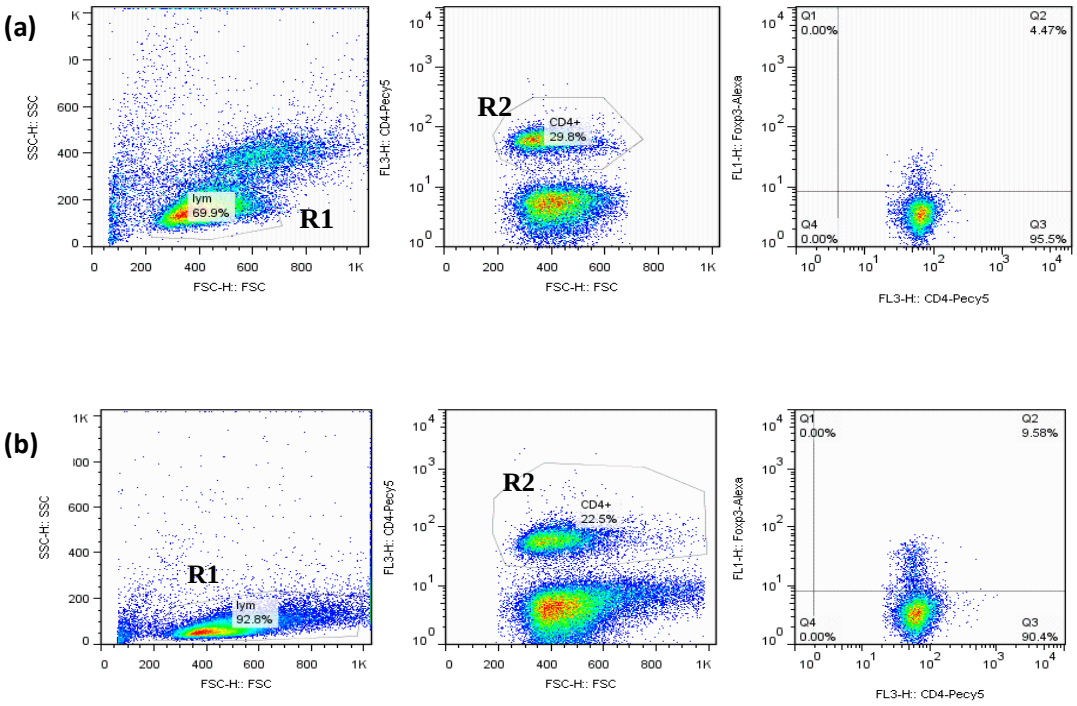


Figure 3.4.1a. A representative figure which shows the gating strategy used for the identification of Foxp3+ Treg cells (CD4+Foxp3+) in freshly isolated tonsillar MNC and PBMC.
(a) PBMC sample **(b)** Tonsillar sample. R1 highlights the lymphocyte population, defined using typical FSC and SSC. Within the lymphocyte population the CD4+ cells are then defined in R2. Finally, the Foxp3+ Treg cells are identified by the gating of the CD4+ population (R2) and the positive staining of Foxp3. This CD4+Fox3+ cell population is defined as the frequency of Treg cells and is shown as a percentage

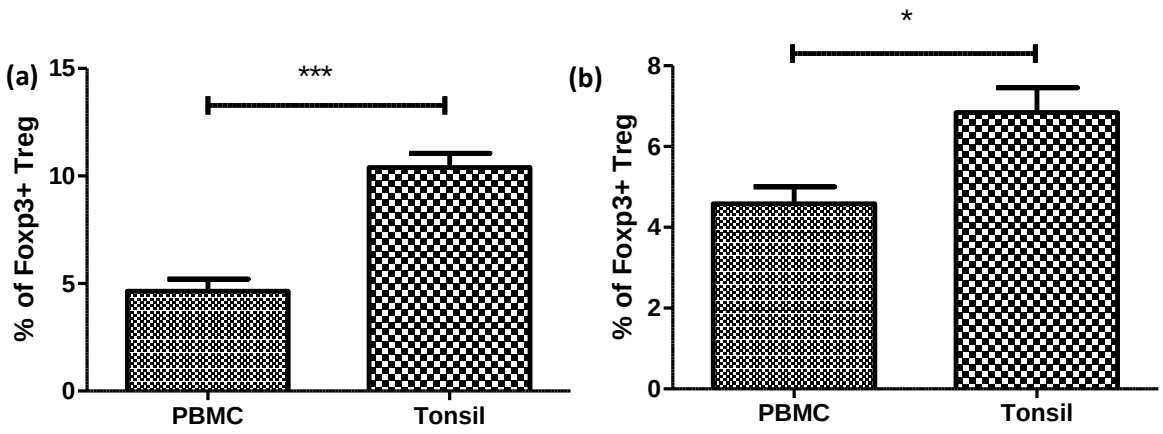


Figure 3.4.1b (a) The frequency of Foxp3+ Treg cells in children tonsillar MNC and PBMC
The frequency of Foxp3+ Treg cells is shown to be significantly higher in tonsillar MNC compared to PBMC. (10.4% in tonsillar tissue samples and 4.6% in peripheral blood samples). (p=<0.0001, n = 15). Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001 **(b)The frequency of Foxp3+ Treg cells in adult tonsillar MNC and PBMC.** The frequency of Foxp3+ Treg cells is shown to be significantly higher in tonsillar MNC compared to PBMC. (6.8% in tonsillar tissue samples and 4.6% in peripheral blood samples). (p= 0.0115, n = 9). Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

3.4.2 The frequency of Tr1 cells in tonsillar MNC and PBMC samples

The frequency of Tr1 cells in tonsillar MNC and PBMC samples was shown by the staining of a combination of surface and intracellular markers. Cells were stained using CD4, CD49b, LAG-3 and IL-10. CD4+CD49b+LAG-3+ cells which were also shown to produce high levels of the intracellular cytokine IL10 were concluded to be Tr1 cells. A representative figure showing the gating strategy used can be found in figure 3.4.2a. Figure 3.4.2b shows that the mean frequency of Tr1 cells in children tonsillar MNC (6.25%) is significantly higher than in PBMC (2.2%). Figure 3.4.2c shows that the mean frequency of Tr1 cells in adult tonsillar MNC (0.97%) is significantly higher than in PBMC (0.33%)

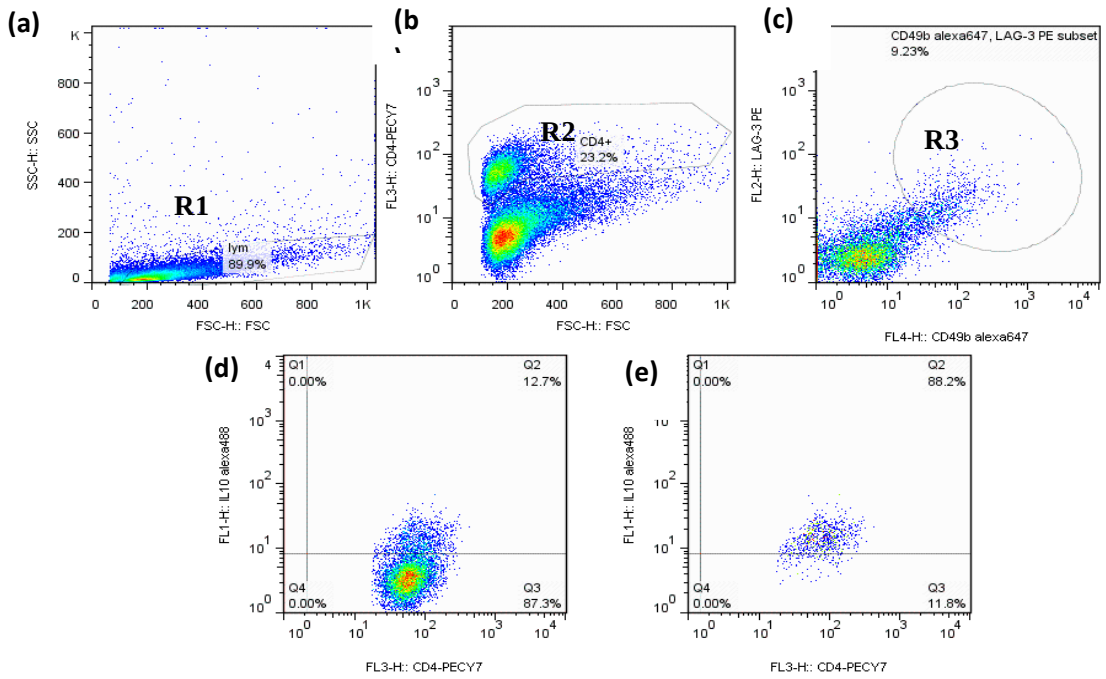


Figure 3.4.2a A representative figure, which shows the gating strategy, used for the identification of Tr1 cell frequency in freshly isolated tonsillar MNC and PBMC.

The lymphocyte population is highlighted in R1, using the typical FSC and SSC configuration. (b) Within the lymphocyte population the CD4+ cells are defined in R2. (c) R3 then highlights the cells within R2 which are both CD49b+ and LAG3+ and are therefore defined as Tr1 cells. (d) IL10 positive cells within the CD4+ lymphocyte population. (e) In order to confirm the cells in R3 are Tr1 cells they are then gated against IL10.

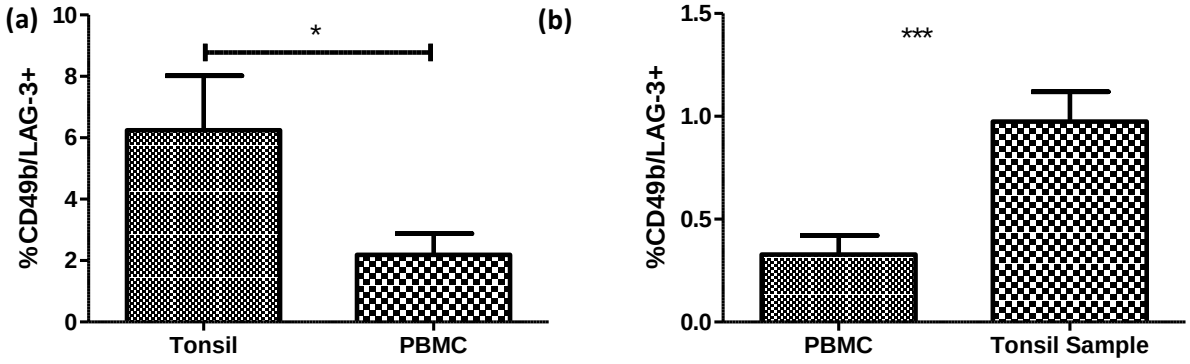


Figure 3.4.2b (a)The frequency of Tr1 cells in children tonsillar MNC and PBMC The frequency of Tr1 Treg cells is shown to be significantly higher in tonsillar MNC compared to PBMC. (6.25% in tonsillar MNC and 2.2% in PBMC) (p=0.0262, n = 7). Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001. **(b)The frequency of Tr1 cells in adult tonsillar MNC and PBMC** The frequency of Tr1 Treg cells is shown to be significantly higher in tonsillar MNC compared to PBMC. (0.97% in tonsillar MNC and 0.33% in PBMC) (p= 0.0002, n = 7). Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

3.4.3 Foxp3+ Treg cell frequency is higher in children tonsillar MNC samples than in adult tonsillar MNC and PBMC samples

The frequency of Foxp3+ Treg cells was measured in both children and adult tonsillar MNC and PBMC to allow a comparison between age groups. The frequency of Foxp3+ Treg cells was measured by staining with CD4 and Foxp3. Figure 3.4.3b shows that the mean frequency of Fox3+ Treg cells in PBMC is no different to in adult samples (4.6% vs 4.6%). However, in tonsillar MNC the mean percentage of Foxp3+ Treg cells is significantly higher in children samples (10.4%) compared to adult samples (6.8%). (Figure 3.4.3b)

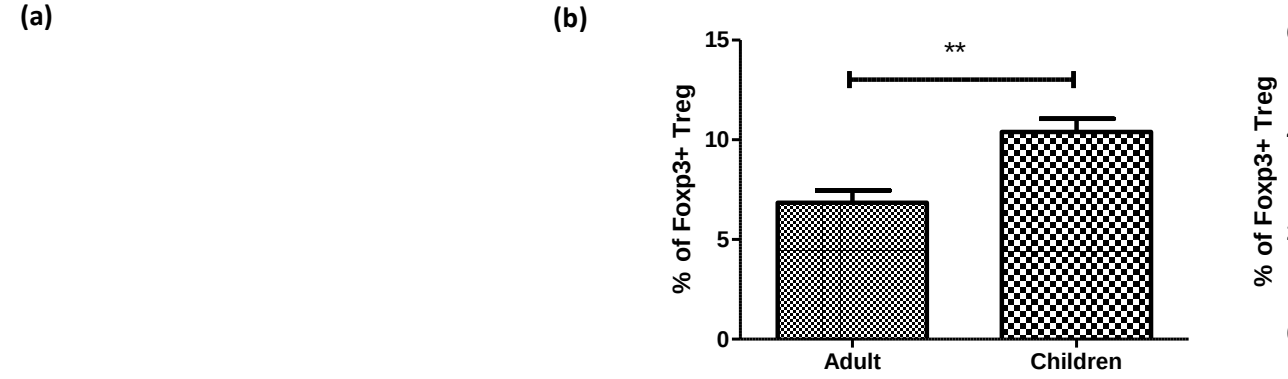


Figure 3.4.3 Frequencies of Foxp3+ Treg cells in adult and children tonsillar MNC and PBMC
(a) The frequency of Foxp3+ Treg cells in tonsillar MNC is shown to be higher in children than in adults (10.4% in children and 6.8% in adults) (p= 0.0017) Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001. **(b)** The frequency of Foxp3+ Treg cells in PBMC is shown to be the same in both adults and children (4.6% for both)

3.4.4 Tr1 cell frequency is higher in children tonsillar MNC samples than in adult tonsillar MNC and PBMC samples

The frequency of Tr1 cells was measured in both children and adult tonsillar MNC to see if any difference in cell numbers was observed in different age categories. Tr1 cells were identified by the staining of CD4, CD49b, LAG-3 and IL-10. A representative example of the cell staining, and gating strategy is shown in figure 4. Figure 3.4.4 shows the mean percentage of Tr1 cells in both children and adult tonsillar MNC samples. The mean

percentage of Tr1 cells in tonsillar MNC is higher in children (6.25%) then in adult samples (0.97%)

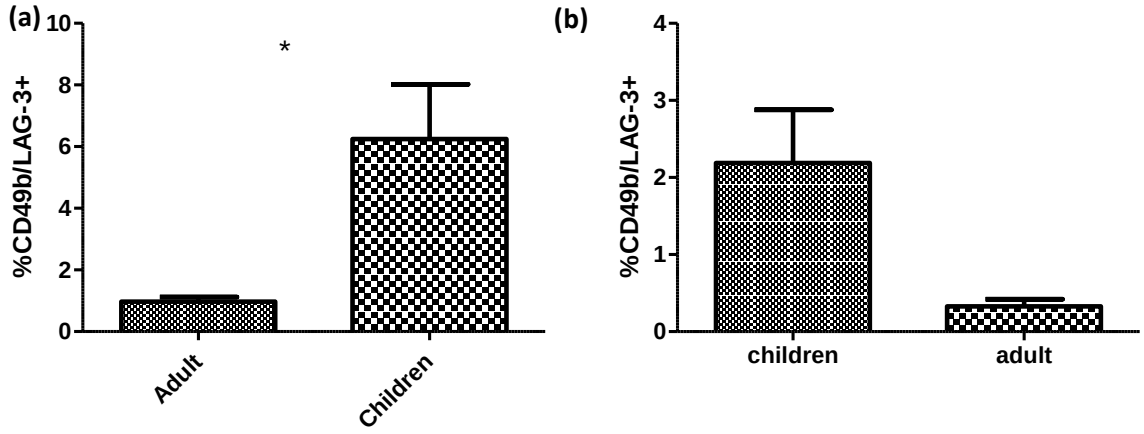


Figure 3.4.4 Frequencies of Tr1 cells in adult and children tonsillar MNC and PBMC samples

(a) The frequency of Tr1 cells in tonsillar MNC is shown to be higher in children than in adults (6.25% in children and 0.97% in adults) (p= 0.0117) Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001. (**b)** The frequency of Tr1 cells in PBMC is shown to be higher in children than in adults (2.2% in children and 0.33% in adults) (p= 0.0477) Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

3.5 Discussion

Data showing frequencies of Tr1 cells in human peripheral blood is limited, and there is currently no data showing frequencies of Tr1 cells in human tonsillar tissue or showing any kind of pattern or relationship between the mucosal nasopharyngeal compartment and the systemic compartment. Data showing the relationship between Foxp3+ Treg cell frequency in the NALT and peripheral blood and the differences seen with age is also limited.

It has been suggested that Treg cells may have an important role to play in mediating the persistence of pneumococcal carriage in the NALT. As such, it is hypothesised that an increased frequency of Treg cells would be observed in tonsillar MNC compared to PBMC. In this study several experiments have been performed to analyse the frequencies of both Foxp3+ Treg and Tr1 cells in adult and children tonsillar MNC and PBMC.

I have shown that adult and children paired tonsillar MNC samples, there are fewer Foxp3+ Treg cells in PBMC samples compared to in tonsillar MNC. (Figure 3.4.1b, Figure 3.4.1c). This suggests that the mucosal compartment of the nasopharynx, which is the location of tonsillar tissue, is more exposed to pathogenic antigen than the systemic system where PBMCs are found. The systemic compartment is a usually sterile site, whereas the nasopharynx is a site of microbial colonisation, so it is consistent with the hypothesis that an increased exposure to microbes in the nasopharynx leads to an increase in T regulatory cells.

We have also shown that the frequency of both Foxp3+ Treg cells in tonsillar MNC and PBMC changes with age. Children samples show much higher frequencies of Foxp3+ Treg cells compared to adult samples (Figure 3.4.3). Young children are frequently exposed to pneumococcus which remains in the nasopharynx without being cleared. It is thought that almost all children are colonised by at least one pneumococcal serotype in their first couple of years of life[5]. The incidence of pneumococcal colonisation has been shown to decrease with age, but it is not uncommon for children to be repeatedly colonised with numerous serotypes[6, 129]. Colonisation at a young age provides immunological memory against pneumococcal antigens which may provide protective effects against colonisation in later life and may thus contribute to the decrease in colonisation events seen with age[259].

These findings suggest that Foxp3+ Treg cells may be responsible for the persistence of carriage seen in young children as a direct result of the activation of Treg cells. Local colonisation occurs within the nasopharynx, so exposure to microorganisms is increased in this compartment when compared to other compartments such as the peripheral blood. A colonisation event could lead to the activation of antigen specific Treg cells which prevent the clearance of the bacteria from the nasopharynx and thus contribute to the persistent carriage of the microbe[90].

It was shown the numbers of Tr1 cells was higher in tonsillar MNC compared to PBMC in both children and adults (Figure 3.4.2b, Figure 3.4.2c). Tr1 cells are thought to differ from naturally occurring Foxp3+ Treg cells that emerge from the thymus in that they are induced from naïve cells when stimulated by antigen in an IL-10 dependent process[106]. They have typically been shown to exist in much lower frequencies than Foxp3+ Treg cells, with research mainly being in peripheral blood samples[260]. To the best of my knowledge this is the first research to investigate Tr1 cells numbers in tonsillar samples of healthy adults and children and to compare Tr1 cell frequencies in 2 separate compartments, the NALT and PBMCs. The pattern of results observed is the same as that seen with Foxp3+ Treg cells, with much higher frequencies observed in the nasopharynx, where colonisation occurs, compared to the peripheral blood which is a sterile site that doesn't encounter pathogenic stimulation. This could suggest that Tr1 cells are also activated or primed by colonisation events by the pneumococcus. Tr1 cells could play a similar role in inhibiting the clearance of the bacteria from the nasopharynx and could play a role in the spread of the bacteria through persistence of carriage.

Tr1 cells were also shown to be found in higher numbers in children samples, where an age associated decrease in cell frequency was observed which inversely correlated with an increase in age (Figure 3.4.4) Again, the number of colonisation events and the length of carriage by pneumococcus has been shown to decrease with age[40]. The adult samples we processed were shown to have a lower percentage of Tr1 cells present compared to the children samples. Tr1 cells could therefore have a role to play in the persistence of carriage seen in children and the lower frequency in adults could be due to the lack of Tr1 cells which allows the clearance of the bacteria.

3.6 Summary

The results gathered here show that that there are significantly higher numbers of Foxp3+ Treg and Tr1 cells in tonsillar MNC than PBMC in both children and adults. This may explain the persistence of carriage which is seen in the nasopharynx. The numbers of Foxp3+ Treg and Tr1 cells is significantly higher in tonsillar MNC from children samples compared to adult samples which could be down to the amount of exposure experienced in early childhood inhibiting the successful clearance of the bacteria from the nasopharynx.

Chapter 4

Foxp3+ Treg cell activation by Pneumococcal Antigens in Human Nasopharynx-Associated Lymphoid Tissues

4.1 Introduction

The results presented in chapter 3 show that there are significant numbers of Foxp3+ Treg cells present in freshly isolated human tonsillar tissue. We hypothesise that stimulation using pneumococcal protein antigens may be able to activate Foxp3+ Treg from freshly isolated tonsillar MNC.

Pneumococcus is a major cause of invasive infectious diseases worldwide, with high morbidity and mortality, particularly in the elderly and very young children[261].

Pneumococcus is harmless when it is at its natural site of colonisation, the nasopharynx, but if it is able to move to other areas of the body such as the lungs or brain it can cause a wide range of serious diseases[4]. Colonisation of the upper respiratory tract is harmless in individuals who are otherwise fit and healthy. But in at-risk individuals such as children under 2 and the elderly the consequences of colonisation can be much more severe. Given that invasive disease cannot occur without colonisation, it has been suggested that a vaccine that targets the nasopharynx and reduces carriage rates would be effective in preventing the spread of bacteria through the community and would reduce the risk of invasive disease

Pneumococcus has a wide variety of virulence factors which contribute to its successful colonisation and infection on individuals. These include the polysaccharide capsule, Pneumolysin and a variety of surface proteins. The polysaccharide capsule is currently used in vaccines against pneumococcus but does have limitations with its narrow serotype coverage and expense. Recently a number of pneumococcal proteins have been investigated for their potential inclusion in a pneumococcal vaccine including pneumolysin and choline binding protein A (CbpA).[12].

Pneumococcal polysaccharide capsule is approximately 200-400 nm thick and forms the outer layer of pneumococcus. Strains of pneumococcus that are lacking in the capsule are avirulent and do not cause disease[39]. The capsule acts to reduce the entrapment of bacteria

in mucosal secretions and acts as a defensive barrier against the binding of complement and other component s which would lead to the phagocytosis of the bacteria[19].

Pneumolysin is cholesterol dependent cytolytic pore forming toxin which is expressed almost universally by all known clinical isolates of pneumococcus[47]. Upon binding to cell membranes, pneumolysin undergoes a conformational change which leads to its insertion in the membrane and the formation of pores. Pneumolysin can activate complement components and also acts through its pore forming ability to directly cause tissue and cellular damage. Clinical isolates which have been found to not express pneumolysin have been shown to be successfully cleared from the lungs suggesting the pneumolysin has an important role in the pathogenicity of pneumococcus[262].

Previous studies have shown that pneumolysin and its toxoids are able to produce protective immunity against both colonisation of pneumococcus and invasive disease[240]. Animal models have shown that mucosal immunisation of the nasopharynx using pneumococcal antigens such as pneumolysin may induce protection against both invasive disease and nasopharyngeal carriage[120, 263]. A reduction in carriage would protect against the spread of bacteria and reduce the likelihood of at-risk individuals developing invasive disease.

Pneumococcal protein antigens have been shown to induce protection against pneumococcus of multiple different serotypes[240, 264, 265]. If they could be used in a successful vaccine that would target all serotypes, the cost of the vaccine would be significantly cheaper than those currently produced, and their effectiveness would also be much more far reaching. Currently only specific serotypes are targeted and although protection against these is good, new serotypes tend to emerge as dominant serotypes. A vaccine containing a combination of pneumococcal protein antigens or a mix of polysaccharide and proteins could be used to confer protection to more pneumococcal

serotypes and would provide wider protection. Also, as protein antigen induced antibody response is T cell dependent, it is likely to be effective in both children and adults.

Previous studies have shown that pneumococcal proteins may be involved in the activation of Foxp3+ Treg cells in tonsillar tissue[90]. Regulatory T cells have an inverse relationship with effector T cells and this relationship is finely balanced to allow the effective clearance of bacteria without severe adverse inflammation occurring to host tissue[129]. Pneumococcal whole cell antigen (WCA) has been shown to promote an increased number of Foxp3+ Treg cells in tonsillar tissue which suggests there is a pneumococcal component which has an effect on numbers of this cell type[90]. To create a successful mucosal vaccine against pneumococcus it is therefore important to understand the relationship between regulatory T cells in tonsillar issue and pneumococcus.

Several different pneumococcal proteins have been studied as potential targets for vaccines[47, 123, 124, 126]. Amongst these, is pneumolysin, a highly conserved protein which causes the formation of pores in cell membranes containing cholesterol. Ply is in the cytoplasm of S. pneumoniae but is released by autolysis[199]. Ply is a major virulence factor, exerting its effect on different cell types including immune cells and epithelial cells. Antibodies against Ply can be found in humans who have been colonised by S. pneumoniae and these antibodies have been shown to passively protect mice that have been challenged with pneumococci[200]. Pneumolysin in its purified form is highly toxic and as such wouldn't be suitable for use in a vaccine targeted to humans. However, Ply can be detoxified which involves its genetic or chemical modification to remove its haemolytic activity. Recently a detoxified genetic mutant of Ply (PlyD1) has been developed which has been used in mouse models and has shown some protection is conferred to the mice involved[202]. PlyD1 has been used in phase 1 human studies and has shown to be safe as well as immunogenic in adults that were involved in the trials

Ply has been studied in mouse models and in natural infections in humans. It has been shown to induce both adaptive and innate immune responses and antibodies against Pneumolysin have been found in individuals infected with S. pneumoniae[200, 203]. There is limited genetic variation of Pneumolysin between different serotypes of S. pneumoniae which suggests it may be a suitable target for inclusion in a vaccine[204].

Pneumolysin has been shown to be essential for maintaining long term asymptomatic carriage in the nasopharynx[126]. Upon administration of a WT strain carriage was maintained for 28 days whereas after administration of PLN-A (pneumolysin deficient mutant) carriage was cleared between 7-14 days after infection. Another study has shown that stimulation of adenoidal MNCs with Ply or F433 leads to proliferation of CD4 T cells[85].

Capsular polysaccharide has been used to vaccinate against pneumococcus since the early 1900's, initially as polysaccharide only vaccines and then in the form of polysaccharide conjugate vaccines[10, 166, 167]. They confer a great deal of protection against the serotypes included in the vaccines, but other serotypes are still able to cause disease and have been shown to increase in prevalence to replace those protected by the vaccine[182, 194]. Furthermore, they are expensive to produce.

Several papers have suggested that pneumococcal components can suppress allergic airways disease and are able to do this through the induction of Treg cells. Using a mouse model of allergic airways disease, Thorburn et al identified that type 3 polysaccharide (T3P) and Pneumolysin when administered together caused an increase in Treg cell numbers in the lungs when compared to a control group[127]. This increase in Treg cells wasn't seen when the pneumolysin and the T3P were administered separately[127]. It has also been demonstrated that a fusion conjugate containing cell wall polysaccharide, Pneumolysin and PsaA delivered alongside cholera toxin nasally protected mice against experimental

colonisation[123]. This combination of polysaccharide and protein could be a promising combination for a vaccine against pneumococcus.

4.2 Aims of the study

In this chapter we have investigated:

1. Whether stimulation using pneumococcal CCS with and without pneumolysin or capsular polysaccharide can activate Foxp3+ Treg cells
2. If the toxoid of Pneumolysin, W433F and purified capsular polysaccharide T3P and 6B can activate Foxp3+ Treg cells in tonsillar MNC.

4.3 Experimental Design:

Tonsillar MNC were stimulated using pneumococcal CCS and other pneumococcal antigens to see if they were able to activate Foxp3+ Treg cells. To assess the ability of the antigens to activate Foxp3+ Treg cells, freshly isolated tonsillar MNC were stimulated for 3 days before intracellular staining and FACS analysis. Foxp3+ Treg cell responses after stimulation were analysed using the measurement of Foxp3 expression.

4.3.1 Human subjects and samples

Samples of Tonsillar tissue were obtained from children undergoing routine adenotonsillectomy between the ages of 2-16 years of age. Any individual who had been exposed to antibiotics for three weeks prior to surgery or were suffering from severe infection or immunodeficiency were excluded from the study. All children involved in the study gave their permission to be involved through informed consent of their parent or guardian.

4.3.2 Pneumococcal concentrated culture supernatant (CCS)

For some of the experiments performed in this chapter, Pneumococcal CCS was prepared and used to stimulate stimulation of tonsillar MNC. Pneumococcal CCS was prepared from the encapsulated type 2 strain D39 (NCTC7466)[238] of S. pneumonia and its isogenic

mutants, one of which was deficient in capsule (Cap-/-) and one of which was deficient in Pneumolysin (Ply -/-) [200]. Pneumococcal CCS was used to stimulate tonsillar MNC for three days, after which the frequency of Foxp3+ Treg cells was analysed by intracellular cytokine staining and FACS analysis. The Pneumococcal CCS was prepared following the method outlined previously by Zhang et al (ref). Bacterial frozen stocks were cultured overnight on blood agar plates (Fisher Scientific, UK) (37°C, 5% CO2). After approximately 18 hours, typical α-haemolytic colonies of *S. pneumoniae* were observed which were then used to inoculate Todd Hewitt Broth (THB) (Oxoid, UK) containing 5% yeast extract. This broth was then cultured overnight (37°C, 5% CO2). The following day, the optical density of the culture was measured at 620nm and then this was checked every 30 minutes until exponential phase of growth was reached (OD of 0.4-0.5 at 620nm). To collect the supernatant, the broth culture was centrifuged at 3000 x g for 30 minutes and the supernatant removed. This was then passed through a 0.45 µm filter followed by a 0.2 µm filter before the final stage of the concentrating process. The pneumococcal culture supernatant was concentrated by centrifuging the sample at 3000 x g for 30 minutes in a Vivaspin15 concentrator (Sartorius Stedim Biotech, Germany). This process was repeated several times to ensure that the sample was concentrated tenfold. Samples were then aliquoted into 1.5 µl microcentrifuge tubes and stored at -80°C until use. The Bradford protein assay was used to determine the concentration of the prepared pneumococcal CCS and the appropriate volume and concentration was then used to stimulate tonsillar MNC in my experiments.

4.3.3 Foxp3+ Treg cell harvesting, staining and analysis

4.3.3.1 Cell stimulation

500 µl of tonsillar MNC suspension at a concentration of 4 x 10^6 was stimulated for 3 days and kept in an incubator at 37°C in 5% CO2. After 3 days, 0.5 µl of a protein transport

inhibitor, Brefeldin A (BFA) was added to each sample before further incubation at 37°C in 5% CO2 for 4 hours. The incubation was then stopped, the plate wrapped in cellophane and left in the fridge overnight.

4.3.3.2 Cell harvest and staining

The following morning cells were harvested from the plated into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02 %PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing 50 µl of a master mix containing 20 µl of CD25 and 5 µl of CD4 were added to each sample and incubated at 4°C for 30 minutes. Cells were then washed using 1 ml of FACS staining buffer at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and incubated for 30 minutes at room temperature. Cells were then washed in permeabilisation buffer followed by centrifugation at 600 x g for 8 minutes before the addition of 50 µl of a master mix containing 15 µl of Foxp3 was added to each sample. This was incubated at room temperature for a further 30 minutes followed by a final was step using 1 ml of permeabilisation buffer per sample for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

4.3.3.3 Foxp3+ Treg cell staining

Foxp3+ Treg cells have an important role in the prevention of auto immune disease and in the maintenance of immune tolerance. Foxp3+ Treg cells possess several surface markers which can be used to distinguish them from other cell types. These include CD25, CTLA-4, CD103 and CD39. Foxp3+ Treg cells also express the intracellular transcription factor Foxp3 and this is a marker specific to both thymic and peripheral Foxp3+ Treg cells.

To determine the presence of Foxp3+ Treg cells, after the 4-hour incubation with BFA, adenotonsillar MNC were harvested from the plates into 1.5 ml Eppendorf tubes and

washed with FACS staining buffer (0.02% PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing, 5 µl of PeCy7 (BD Bioscience) conjugated CD4 was added to each sample and before they were incubated at 4°C for 30 minutes. Cells were then washed using 1 ml of FACS staining buffer, followed by centrifugation at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and they were incubated for 30 minutes at room temperature. Cells were then washed, followed by centrifugation at 600 x g for 8 minutes in permeabilisation buffer before the addition of 15 µl of Alexafluor 647 (BD Bioscience) conjugated Foxp3 to each sample. This was incubated at room temperature for a further 30 minutes, followed by a final wash step using 1 ml of permeabilisation buffer per sample for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

4.3.4 Statistical analysis

Data were analysed using GraphPad Prism version 5. Differences between two groups, for example adults and children, were analysed by Student's t test. Differences between paired samples for example stimulated tonsil and stimulated blood samples, were analysed by paired t (parametric) or Wilcoxon matched-pairs signed rank (non-parametric) test.
A p value of <0.05 was taken as a level of statistical significance.

4.4 Results

4.4.1 Gating strategy for Foxp3+ Treg cells

Foxp3+ Treg cells were defined as CD4+Foxp3+ cells when stained using intracellular staining (Figure 4.4.1). The lymphocyte population was defined in R1 using the typical FSC and SSC distribution (A). Within the lymphocyte population, CD4+ cells were then gated within R2 (B). Foxp3+ Treg were then defined from the CD4+ T cells as a percentage of Foxp3+ cells in (c).

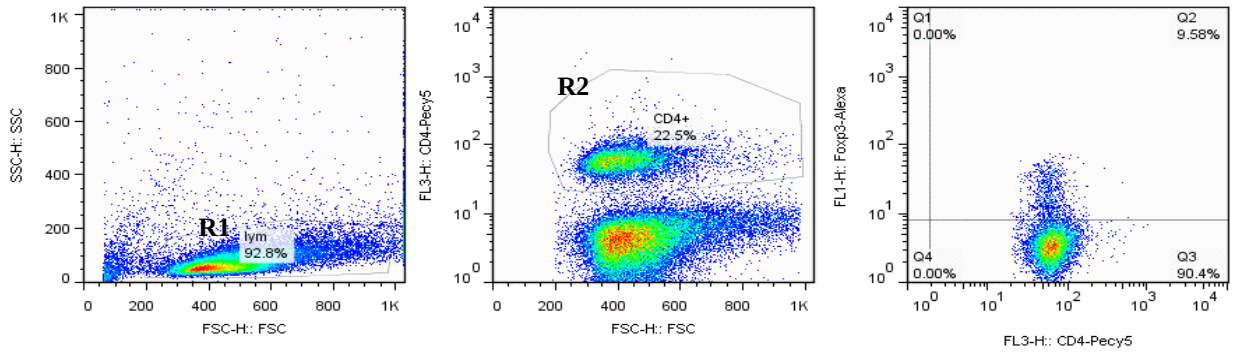


Figure 4.4.1. A representative figure which shows the gating strategy used for the identification of Foxp3+ Treg cells (CD4+Foxp3+) in freshly isolated tonsillar MNC

R1 highlights the lymphocyte population, defined using typical FSC and SSC (A). Within the lymphocyte population the CD4+ cells are then defined in R2 (B). Finally, the Foxp3+ Treg cells are identified by the gating of the CD4+ population (R2) and the positive staining of Foxp3. This CD4+Fox3+ cell population is defined as the frequency of Treg cells and is shown as a percentage in CD4+ T cell (C).

4.4.2 Determination of optimal doses of pneumococcal CCS to be used for the activation and induction of Foxp3+ Treg cells in human tonsillar MNC

D39 WT pneumococcal CCS and its isogenic mutants deficient in either pneumolysin (Ply -/-) or capsular polysaccharide (Cap -/-) were used in experiments looking at the activation of Foxp3+ Treg cells. To determine the optimal dose of pneumococcal CCS to use, a dose response curve of WT pneumococcal CCS was created. The protein concentration of

pneumococcal CCS was measured using the Bradford protein assay and involved the use of Bradford protein dye reagent (Sigma) following manufacturer's instructions. The principle of this assay is to compare sample dilutions against a known standard curve to work out the concentration of your sample. The standard curve used is a Bovine Serum Albumin (BSA) standard curve. A stock concentration of 2 mg/ml of BSA was serially diluted (two-fold) in sterile PBS five times to give the BSA curve. Samples were diluted serially (ten-fold) three times. 5 µl of sample or BSA standard was added to the appropriate well of a 96-well Costar plate and 250 µl of Bradford reagent was then added. The plate was incubated in the dark for 5 minutes before absorbance was read at 595nm using the ThermoElectron MultiSkan (Opsys MR, Thermo labsystems, UK). A standard curve was produced from the readings of the BSA dilutions and this was used to work out the concentrations of the samples using DeltasoftPC microplate reader software. (Biometallics Inc., USA). Figure 4.4.2 summarises the results and shows that the optimal protein concentration of pneumococcal CCS to use for Foxp3+ Treg cell activation is 1 µg/ml. A concentration of 0.5 µg/ml does not stimulate Foxp3+ Treg cells much more than a negative unstimulated control and higher concentrations appear to have a toxic effect on the cells. 1 µg/ml is the concentration of pneumococcal CCS that was used for all Foxp3+ Treg cell activation experiment.

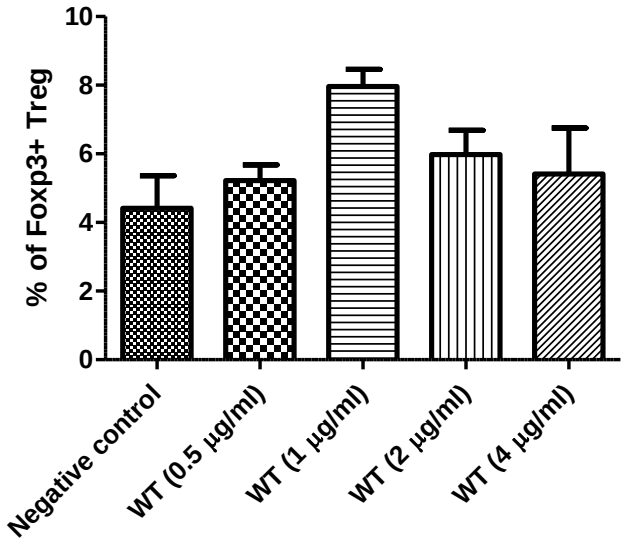


Figure 4.4.2. Dose curve of WT pneumococcal CCS for the stimulation of Foxp3+ Treg cells
Stimulation of Foxp3+ Treg cells with WT pneumococcal CCS shows the optimal protein concentration to be 1 µg/ml.

4.4.3 Determination of optimal doses of W433F, T3P and 6B to be used for the activation of Foxp3+ Treg cells in human tonsillar MNC

The toxoid of pneumolysin W433F and purified capsular polysaccharide types T3P and 6B were used in experiments looking at the activation of Foxp3+ Treg cells. To determine the optimal concentrations to use for these experiments, dose response curves for all three were made. Figure 4.4.3 shows the dose curves for W433F (a), T3P (b) and 6B (c) respectively. The dose curve for pneumolysin toxoid W433F shows the optimal dose for stimulation is 10 ng/ml (n=2). For both T3P and 6B the optimal dose for stimulation is 1µg/ml (n=2). These optimum concentrations were used in all experiments looking at the activation of Foxp3+ Treg cells by W433F, T3P and 6B.

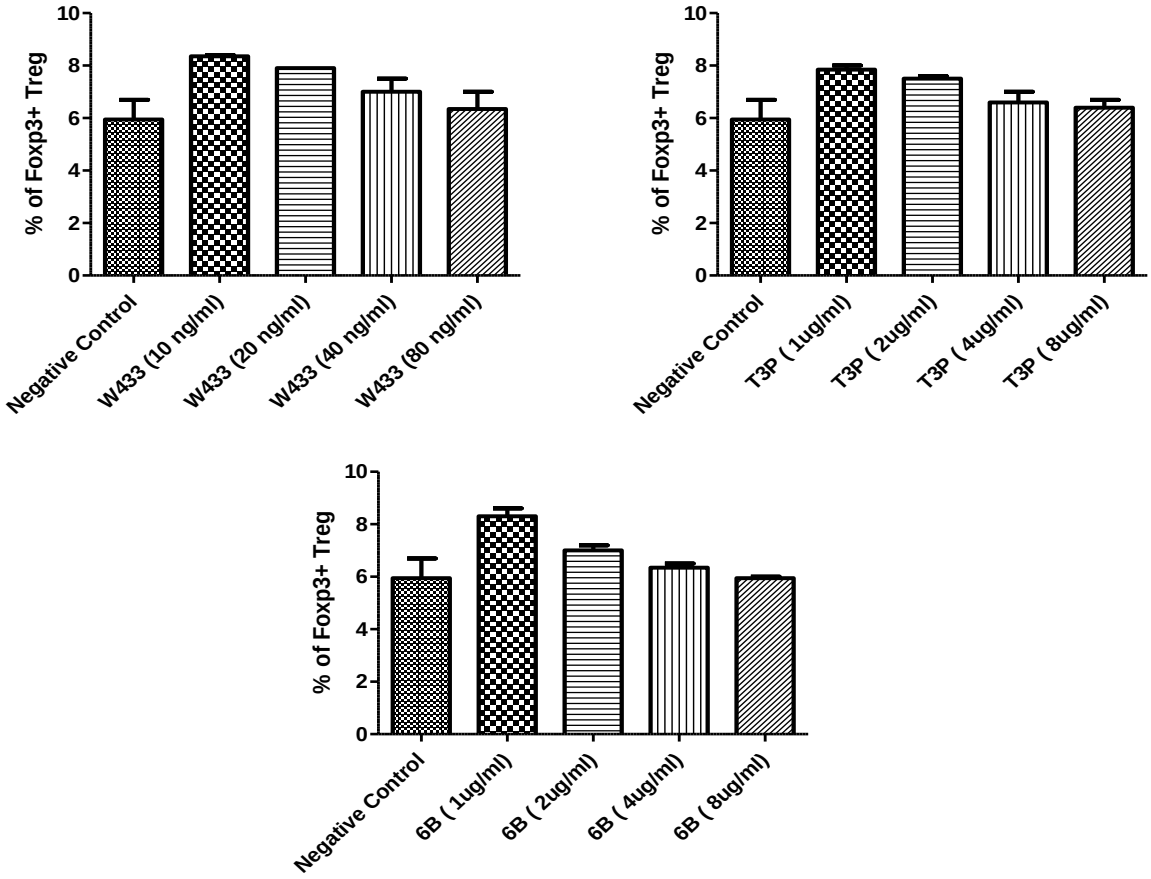


Figure 4.4.3 Dose response curves for W433F, T3P and 6B for Foxp3+ Treg cell stimulation
(a) The percentage of Foxp3+ Treg cell activation by W433F is shown to be optimal at 10 ng/ml with higher concentrations seeming to have a toxic effect on the cells. **(b)** The frequency of Foxp3+ Treg cell activation by T3P is shown to be optimal at 1µg/ml with higher concentrations seeming to have a toxic effect on the cells. **(c)** The frequency of Foxp3+ Treg cell activation by 6B is shown to be optimal at 1µg/ml with higher concentrations seeming to have a toxic effect on the cells.

4.4.4 Stimulation of tonsillar MNC with pneumococcal CCS deficient in pneumolysin (Ply) or capsular polysaccharide show lower Treg cell activation compared to stimulation with wild type pneumococcal CCS

Freshly isolated tonsillar MNC were stimulated using WT, Ply-/- and Cap-/- pneumococcal CCS in order to determine whether the absence of specific key pneumococcal virulence factors (pneumolysin and capsular polysaccharide) had an effect on the activation of Foxp3+ Treg cells. Pneumococcal CCS was used to stimulate freshly isolated tonsillar MNC at a concentration of 1 µg/ml as outlined in figure 4.4.2. Figure 4.4.4 shows that D39WT pneumococcal CCS stimulation of tonsillar MNC leads to the activation of significantly more

Foxp3+ Treg cells (9.47%) compared to the unstimulated negative control (4.86%). When Ply-/- CCS and Cap -/- CCS is used for stimulation the percentage of Foxp3+ Treg cell activation is significantly lower (6.42% and 6.36% respectively)

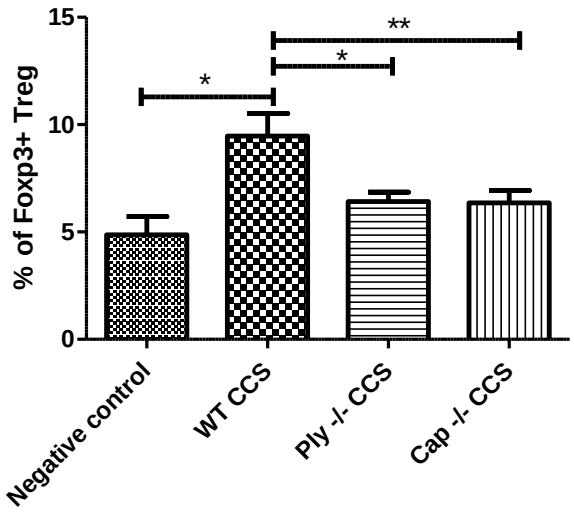


Figure 4.4.4 The frequency of Foxp3+ Treg cell activation after stimulation with pneumococcal CCS
The frequency of Foxp3+ Treg cells is shown to be significantly higher in tonsillar MNC upon stimulation with WT CCS compared to unstimulated negative control (4.86% for the negative control and 9.47% when stimulated with WT CCS). ($p = 0.0156$, $n = 6$). Ply -/- CCS shows significantly less activation compared to the WT CCS (6.42% compared to 9.47%) ($p = 0.0233$, $n = 6$). Cap -/- CCS shows significantly less activation compared to WT CCS (6.36% compared to 9.47%) ($p = 0.0042$ $n = 6$) Mean + SEM are shown *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.4.5 Activation of Foxp3+ Treg cells in tonsillar MNC by pneumolysin toxoid W433F and purified capsular polysaccharide (T3P and 6B)

Results from stimulation of freshly isolated tonsillar MNC with pneumococcal CCS deficient in key pneumococcal virulence factors suggest that pneumococcal pneumolysin and polysaccharide capsule may be important for the activation of Foxp3+ Treg cells in human tonsillar MNC (Figure 4.4.4). To confirm this, human tonsillar MNC were stimulated using purified toxoid of pneumolysin (W433F) and capsular polysaccharide types T3P and 6B.

Freshly isolated tonsillar MNC were stimulated with W433F, T3P and 6B. Data shown in figure 4.4.5 shows that compared to a medium control with no stimulation (5.28%), the stimulation of cells with W433F elicited an increase in Foxp3+ Treg cells (7.41%). The stimulation of tonsillar MNC with both T3P and 6B also showed an increase in Foxp3+ Treg cells (7.78% and 7.65%) compared to the unstimulated medium control (5.28%). Tonsillar MNC were also stimulated using a combination of W433F and T3P and W433F and 6B. The stimulation of tonsillar MNC with W433F and T3P shows significantly more Foxp3+ Treg cell activation (8.72%) compared to an unstimulated control (5.28%) but there is no significance when compared to just W433F and T3P alone. The combination of 6B and W433F shows no significance in Foxp3+ Treg cell activation compared to a negative control or to W433F or 6B alone.

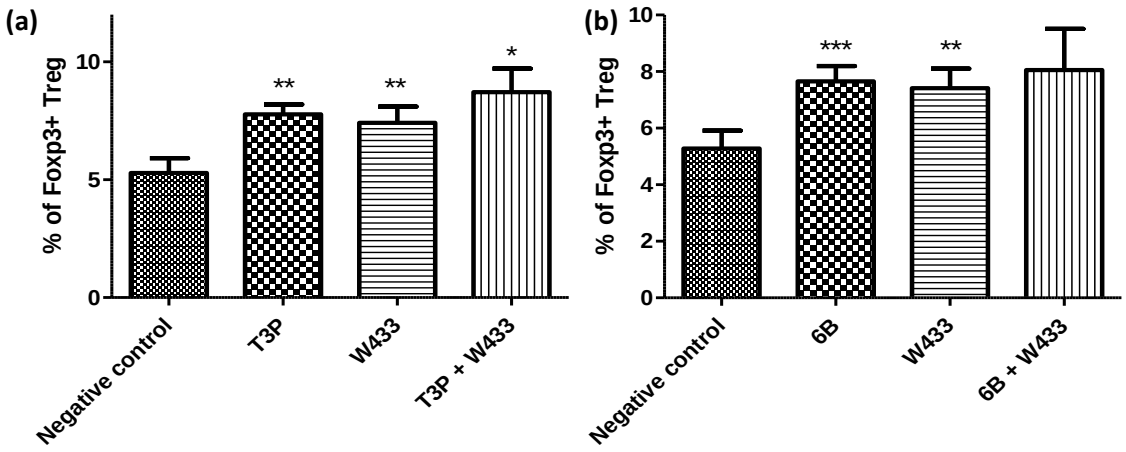


Figure 4.4.5 The frequency of Foxp3+ Treg cell activation after stimulation with pneumococcal capsular polysaccharide (T3P and 6B) and toxoid of pneumolysin W433F

(a) The frequency of Fox3+ Treg cells after stimulation with purified pneumococcal capsular polysaccharide T3P is significantly higher when compared to an unstimulated negative control (7.78% compared to 5.28%, p = 0.0065, n = 9). The frequency of Foxp3+ Treg cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (7.41% compared to 5.28%, p = 0.0037). Stimulation using a combination of T3P and W433F shows a significant increase in Foxp3+ Treg cell frequency compared to a negative control (8.72 compare to 5.28, p = 0.0115, n = 9) but no significant difference compared to T3P and W433F alone. **(b)** The frequency of Fox3+ Treg cells after stimulation with purified pneumococcal capsular polysaccharide 6B is significantly higher when compared to an unstimulated negative control (7.65% compared to 5.28%, p = 0.0007, n = 9). The frequency of Foxp3+ Treg cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (7.41% compared to 5.28%, p = 0.0037). A combination of 6B and W433F does not show any significant increase compared to a negative control or compared to 6Bcap and W433F alone. Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

4.5 Discussion

There is limited data about the role of regulatory T cells and their relationship with pneumococcus during colonisation of the nasopharynx. Data relating to human samples is particularly limited. A previous study showed the pneumococcal whole cell antigen (WCA)

was able to induce Foxp3+ Treg cell proliferation in tonsillar MNCs which suggests there are pneumococcal protein/s which could have a role to play in the induction of these cells[90]. Currently, it remains unclear which pneumococcal proteins may have a role in the activation or induction of regulatory T cells in in human nasopharynx-associated tissue (NALT). The identification of such protein/s would be useful information to further our understanding on the role of Treg in pneumococcal carriage and on novel vaccination strategies against pneumococcal disease.

As previously mentioned, current pneumococcal vaccines target pneumococcal polysaccharide capsule. These vaccines have proven to be effective at protecting individuals against serotypes included in the vaccine. Currently there are over 90 known serotypes of pneumococcus, each classified based on their different polysaccharide capsules. Current conjugate vaccines target 7 or 13 of these but this still leaves a gap with the clear majority of serotypes not being included in the vaccine, largely due to the high cost and technical complexity of producing vaccines with more serotypes included.

This has led to pneumococcal vaccine research becoming more focussed on identifying pneumococcal proteins which could be included in a vaccine that would then be effective against all known serotypes[266, 267]. Pneumococcal proteins including pneumolysin and choline binding protein A have been studied to see if they could be used in a vaccine[240].

This study has shown that in human tonsillar tissue, pneumolysin is able to activate pre-existing Foxp3+ regulatory T cells. Given that pneumolysin is a key virulence factor of pneumococcus, it is important to understand the interactions between it and cells of the immune system. Before it could be included in a vaccine it would be necessary to understand exactly how pneumolysin affects the delicate balance of the immune system. Regulatory T cells are important for preventing the uncontrolled expression of effector T cells which if unchecked could lead to severe host inflammation so understanding how

these cells are activated is very important. Regulatory T cells have also been shown to be important in the carriage of pneumococcus and in causing the persistence of bacteria in the nasopharynx[90, 126].

In this study we investigated the importance of pneumolysin and capsular polysaccharide in the activation of Foxp3+ Treg cells. My first experiment involved the use of pneumococcal CCS with and without pneumolysin and capsule to determine their importance in Foxp3+ Treg cell activation. D39 WT strain of pneumococcal CCS showed significantly higher activation of Foxp3+ Treg cells compared to both mutant strains used. The first mutant strain was pneumolysin (Ply) deficient (Ply -/-) and the second was capsule deficient (Cap -/-). These initial results suggested that both pneumolysin and capsular polysaccharide could be important for the activation of Foxp3+ Treg cells.

After the initial findings using pneumococcal CCS I wanted to look at whether purified capsular polysaccharide and the toxoid of pneumolysin, W433F, could activate Foxp3+ Treg cells compared to a negative control. My results show that W433F and purified capsular polysaccharide are able to activate pre-existing Foxp3+ Treg cells to significantly higher levels compared to an unstimulated negative control. Two different types of capsular polysaccharide were used, T3P and 6B and both were shown to activate Foxp3+ Treg cells compared to the negative control. Capsular polysaccharide is currently used in pneumococcal vaccines and we know it is effective at preventing pneumococcal disease. In my results, pneumolysin has been shown to be effective at activating Foxp3+ Treg cells.

The pre-existing Foxp3+ Treg cells detected in tonsillar tissue are likely to be those which are primed due to previous pneumococcal colonisation events in the nasopharynx. The ability of both W433F and both capsular polysaccharides used to activate these cells suggests that both the polysaccharide capsule and pneumolysin are able to activate these cells and therefore play a role in the carriage of pneumococcus in the nasopharynx.

The ability of pneumolysin to activating pre-existing Foxp3+ Treg cells suggests that this pneumococcal protein does specifically influence the immune response generated in response to pneumococcal exposure. Understanding the precise mechanism of these interactions and its effects on other immune cells is key to further research, which aims to include this protein in any future vaccines. It is interesting to note that pneumolysin is as effective at capsular polysaccharide at activation of Foxp3+ Treg cells and that the combination of both capsular polysaccharide and pneumolysin does not lead to enhanced Foxp3+ Treg cell activation. Capsular polysaccharide is already effective at providing protection against pneumococcal disease and if pneumolysin is as effective, it could be even more successful in conferring protection due to its presence on all known clinical pneumococcal isolate. In addition to this, my research has focussed on the interactions between pneumococcus and Foxp3+ Treg cells in the nasopharynx. Given that the nasopharynx is the site of pneumococcal colonisation, that prolonged carriage of the bacteria leads to spread throughout the community and that disease cannot occur without colonisation, the nasopharynx could be a potential target for a future vaccine that directly works at the mucosal site rather than through the blood. Again, it is important to understand the balances and intricacies of how any potential vaccine would work and how it would affect the hosts' natural response, but this is certainly an interesting and potentially rewarding area of research which could have very fruitful results in the future.

The overall findings in this study support the hypothesis that both pneumococcal polysaccharide and pneumococcal proteins have an important role in the activation of Foxp3+ Treg cells in the nasopharynx. They suggest that in the mucosal tissue of the nasopharynx pneumolysin and capsular polysaccharide can influence these regulatory cells, which leads to persistence of carriage and the delay in pneumococcal clearance.

4.6 Summary

The results presented in this study suggest certain pneumococcal components may have a role in the activation of Foxp3+ T regulatory cells in human NALT. Data suggests that both Pneumolysin and capsular polysaccharide are both able to activate Foxp3+ Treg cells and as such they may contribute to the carriage of pneumococcus in the nasopharynx.

Chapter 5

Tr1 cell activation and induction by Pneumococcal Antigens in Human Nasopharynx-Associated Lymphoid Tissues

5.1 Introduction

The results presented in chapter 1 show that tonsillar tissue contains significant numbers of type 1 regulatory (Tr1) cells. This chapter aims to determine whether specific pneumococcal components such as pneumolysin and capsular polysaccharide can activate or induce Tr1 cells in tonsillar MNC.

Pneumococcus is major cause of morbidity and mortality worldwide, particularly amongst at risk groups including the very young, the elderly and people who are immunocompromised with approximately 1 million children under the age of 5 years of age dying annually[2]. Carriage of the bacteria in the nasopharynx is common, especially in young children who are regularly in contact with one another. At the site of colonisation, the bacterium is usually harmless and doesn't cause disease. However, if it can move from the nasopharynx into other areas of the body such as the lungs or brain it can cause serious infection such as meningitis, septicaemia and pneumonia and is also responsible for many otitis media in young children[2]. In addition, carriage of the bacteria within the nasopharynx is an important source of the spread of infection within a community, with carriage rates reaching 90% in young children[4]. Given that colonisation is a pre-requisite for disease and carriage of the bacteria allows it to be spread throughout the community, a vaccine that targets the site of colonisation, the nasopharynx, could be an effective strategy in reducing the spread of bacteria and in preventing invasive disease[240].

The activation of CD4+ T cells in response to antigenic stimulation results in the generation of effector CD4+ T cells which can then exert their effects at the site of the pathogen invasion. As well as the activation of effector cells, memory cells are also produced upon stimulation by foreign antigen. These memory cells are then sequestered in lymphoid tissue to exert their effects if stimulated by that specific antigen later. It is because of these

memory cells that secondary challenge by the same antigen produces a much more rapid immune response.

Type 1 regulatory T (Tr1) cells are an inducible subset of regulatory T cells that play a role in promoting and maintaining tolerance[103]. The secretion of high levels of IL-10 is the main mechanism by which Tr1 cells exert their effects[268]. They also kill myeloid cells by the secretion of Granzyme B[98]. Prior to 2013, the main identifying method for Tr1 cells has been through their cytokine profile. Tr1 cells secrete a lot of IL-10 and minimal amounts of IL-4 and IL-17 which distinguishes them from Th2 and Th17 cells[98]. Tr1 cells also secrete IL-2 and depending on the local cytokine environment can secrete IFN-y. In 2013, Roncarolo et al showed that the co-expression of cell surface markers CD49b and LAG-3 could be used to identify Tr1 cells and for the purposes of this study it was the co-expression of these cell surface markers that was used to identify the numbers of Tr1 cells present[1].

The generation of Tr1 cells from naïve T cells was first described by Groux et al[105]. They showed that naïve T cells from OVA TCR-transgenic mice repeatedly stimulated with OVA and IL-10 differentiated into T cells with a unique cytokine profile distinct from Th2 and Th1 cells[105]. More recently these Tregs have been shown in humans[268]. These cells have been shown to have immunosuppressive properties that can prevent the development of T cell mediated autoimmunity[269]. Tr1 can control the activation of naive and memory T cells and suppress Th1 and Th2 mediated immune responses[270, 271]. The suppressive effects of Tr1 cells are reversed by blocking antibodies against IL-10, showing that the inhibitory capacity of Tr1 cells is mainly mediated through production of IL-10[272].

Tr1 cells are induced when CD4+ T cells are activated by antigen in the presence of IL-10.[106] It had been difficult to distinguish these cells from other cell types, relying on characterisation using its unique cytokine profile. Tr1 cells are distinguished from Th1 cells by expression of minimal IL4 and down regulation of GATA3 expression[1]. Tr1 cells lack IL-17

and express low levels of RORC which distinguishes them from Th17 cells. Low levels of IL-2 and IFN-γ which distinguishes them from CD25+Foxp3+ Treg cells. It has recently been shown that there are cell surface markers characteristic of Tr1 cells which allow for them to be differentiated from other regulatory T cells[1]. Tr1 cells are CD4+CD49b+LAG-3+[1]. This ability to differentiate Tr1 cells from other cells means further study of this cell type is possible.

IL-10 is an immunomodulatory cytokine that plays an essential role in regulation of the immune system in a variety of ways. It is responsible controlling inflammatory responses by down regulating inflammatory cytokines, induces tolerance and is important in the down regulation of other immune responses[107]. IL-10 is indispensable for driving the differentiation of Tr1 cells but other cytokines are also involved.

During infection IL-10 inhibits the activity of a variety of different cell types including Th1 cells and macrophages. These cells are essential for the clearance of pathogens but can also be responsible for causing tissue damage which is why they must be regulated[108]. For this reason, IL-10 can be responsible for inhibiting clearance of pathogens and preventing inflammation associated immunopathology.

The conclusion that Tr1 cells can be identified by the co expression of CD49b and LAG-3 has allowed for them to be studied in much more detail than previously[1]. LAG-3 is a protein antigen expressed on T cells and NK cells after cell activation. It is a CD antigen given the name CD223[111]. The expression of LAG-3 on the surface of activated T cells is upregulated by IL-2, IL-7 and IL-12 and its expression leads to the production of IFN-γ. LAG-3 has also been shown to be expressed on activated B cells but not when the B cell is activated solely by a TLR agonist[112]. LAG-3 expression on B cells could therefor serve as a marker for B cells that have been activated by T cells.

LAG-3 is a multifunctional protein and can affect a variety of different cell types. LAG-3 is a ligand for MHC class 2. The interaction between LAG-3 and MHC class 2 controls CD4+ T cell responses and leads to the down regulation of antigen specific CD4+ T cell proliferation and cytokine secretion[113].

LAG-3 has been shown to be important for maximal function of regulatory T cells. Antibodies against LAG-3 have been shown to inhibit suppression by induced Treg cells both in vitro and in vivo[115]. LAG-3 knockout mice have been shown to have regulatory T cells with reduced regulatory function[115].

CD49b (cluster of differentiation 49b) is a protein encoded by the CD49b gene. CD49b is the α2 integrin subunit of very late activation antigen 2 (VLA-2)[1]. Integrins are integral membrane glycoproteins composed of an alpha and a beta subunit and are found on a variety of cell types including T cells and NK cells[116].

Current vaccines against pneumococcus are centred on targeting the polysaccharide capsule. This is one of the main virulence factors of pneumococcus, with strains that lack a polysaccharide capsule being unable to cause invasive disease as they become trapped in cell surface mucous and are unable to get free[41]. The original vaccines against pneumococcus were not very effective, being unable to produce an immune response in children as they act in a T cell independent fashion[4]. This led to the creation of pneumococcal polysaccharide conjugate vaccines which include polysaccharide conjugated to protein and are effective in young children. However, these vaccines are still limited by their narrow serotype coverage and expense. Vaccines contain polysaccharide against the most common invasive serotypes but not against all as this would be far too expensive. This means that pneumococcal infection caused by serotypes not contained within the vaccine became more dominant[28]. Recently, vaccine research has moved on from looking at polysaccharide vaccines towards looking at the potential use of pneumococcal proteins

that are well conserved amongst different serotypes in a vaccine. There are several pneumococcal proteins which are well conserved among pneumococcal serotypes such as pneumolysin and these are considered promising vaccine candidates[273].

One of the key virulence factors of pneumococcus is the cholesterol dependent cytolytic pore forming toxin pneumolysin[274]. Pneumolysin acts by forming pores in the cell membrane causing the cell to lyse. It does this by binding to cells which then undergoes a conformational change leading to its insertion in the membrane and the formation of pores. Pneumolysin is expressed almost universally in all known clinical isolates of pneumococcus with isolates that do not express pneumolysin being shown to be effectively cleared from lungs which suggests it has an important role to play in pneumococcal virulence and pathogenesis[47].

Nasopharyngeal colonisation is very common in young children before natural immunity can develop. In the UK colonisation in children less than 3 years of age is between 40-50%[117] but in African children this can reach 90-100%[118, 119]. By the age of 3 most children have developed natural immunity against specific pneumococcal antigens. This natural immunity is responsible for the clearance of the pneumococcus from the nasopharynx before carriage status can be achieved. However, despite this natural immunity carriage can still occur throughout life and it is thought that Treg cells could have a role in this.

Due to the success of vaccines targeted to the polysaccharide capsule of the pneumococcus, it has classically been thought that protection against it was due to an antibody-based response[120]. However, more and more publications are now suggesting an important role for CD4+ T cell immunity in protection against the pneumococcus. MHC-II knockout mice which lack the ability to induce cell-mediated immunity through the presentation of antigen show prolonged carriage [120] suggesting CD4+ T cells have an important role to play in pneumococcal carriage rather than antibody-based responses.

The role of Foxp3+ Treg cells in the carriage of pneumococcus has been studied and a role for these regulatory cells has been sugeested[90, 129, 273]. However, the role of Tr1 cells during pneumococcal colonisation and their potential role I carriage of the bacteria has not been studied.

This study looked at the induction and activation of Tr1 cells in human tonsillar tissue by pneumococcus to see if Tr1 cells could have a role to play in the carriage of bacteria.

5.2 Aims of the study

In this chapter we have investigated:

1) Whether stimulation of freshly isolated tonsillar MNC with pneumococcal CCS, both wild type and deficient in pneumolysin or capsule, causes the activation of pre-existing Tr1 cells.
2) If pneumococcal CCS can induce Tr1 cells from naïve CD4+ T cells in CD45RO+cell-depleted tonsillar MNC.
3) If the toxoid of Pneumolysin, W433F and purified capsular polysaccharide T3P and 6B can activate Tr1 cells in tonsillar MNC.
4) If the toxoid of Pneumolysin, W433F and purified capsular polysaccharide T3P and 6B can induce Tr1 cells from naïve T cells in CD45RO+ depleted tonsillar MNC.

5.3 Experimental Design

Tonsillar MNC were stimulated using pneumococcal concentrated culture supernatant (CCS) derived from wild type (WT) D39 pneumococcus or its pneumolysin (Ply-/-) deficient strains. For activation experiments, tonsillar MNC were directly stimulated whereas for induction experiments tonsillar MNC were first depleted of memory CD45RO+ T cells using MACS cell separation. Freshly isolated tonsillar MNC s were cultured for 3 days before the activation of Tr1 cells was measured using intracellular cytokine staining followed by flow cytometric analysis. To assess the induction of Tr1 cells from naïve CD4+ T cells, CD45RO+

cells were first depleted before the cells were again cultured for 3 days followed by FACS analysis. Tr1 cell responses were analysed by the measurement of the intracellular cytokine IL-10 and the co expression of the extracellular markers CD49b and LAG-3.

5.3.1 Human subjects and samples

Samples of tonsillar tissue were obtained from children undergoing routine adenotonsillectomy between the ages of 2-16 years of age. Any individual who had been exposed to antibiotics for three weeks prior to surgery or were suffering from severe infection or immunodeficiency were excluded from the study. All children involved in the study gave their permission to be involved through informed consent of their parent or guardian.

5.3.2 Pneumococcal concentrated culture supernatant (CCS)

Pneumococcal CCS was used for the stimulation of tonsillar MNC in this experiment. Pneumococcal CCS was prepared from the encapsulated type 2 strain D39 of S. pneumonia and its isogenic mutants, one of which was deficient in capsule (Cap-/-) and one of which was deficient in Pneumolysin (Ply -/-).

To prepare Pneumococcal CCS, bacterial frozen stocks were cultured overnight on blood agar plates (Fisher Scientific, UK) (37°C, 5% CO2). After about 18 hours, typical α-haemolytic colonies of *S. pneumoniae* were observed. Several colonies were then used to inoculate Todd Hewitt Broth (THB) (Oxoid, UK) containing 5% yeast extract which was then cultured overnight (37°C, 5% CO2). The following day, the optical density of the culture was measured at 620nm and then this was checked every 30 minutes until exponential phase of growth was reached (OD of 0.4-0.5 at 620nm). The broth culture was centrifuged at 3000 x g for 30 minutes and the supernatant removed. The supernatant was then passed through a 0.45 µm filter followed by a 0.2 µm filter. Concentration of the pneumococcal culture supernatant was then achieved by centrifuging the sample at 3000 x g for 30 minutes in a Vivaspin15 concentrator (Sartorius Stedim Biotech, Germany). This process was repeated

several times to ensure that the sample was concentrated tenfold. Samples were then aliquoted into 1.5 µl microcentrifuge tubes and stored at -80°C until use.

5.3.3 Depletion of CD45RO+ cells from Tonsillar MNC

For Tr1 cell induction experiment, to remove any pre-existing activated Treg cells, CD45RO+ cells were first depleted from the cell culture. CD45RO is expressed on a number of different cell types. It is found on both memory CD4+ and CD8+ T cells as well as being present on CD4+ effector T cells, macrophages and monocytes. Previously activated and induced Treg cells are CD45RO+, whereas naïve Treg cells are CD45RA+. Depletion of CD45RO+ cells will remove any previously activated Treg cells leaving a naïve population that have never encountered antigen and become activated.

To deplete cells, approximately 50 x 10^6 cells were taken from tonsillar MNC mix and re-suspended in 400 µl (80µl/107 cells) of depletion buffer (0.5% PBS BSA). To this, 100µl (20µl/107 cells) of CD45RO magnetic beads (Miltenyi Biotech) were added. The cell suspension was thoroughly vortexed followed by incubation at 4°C for 15 minutes. Following incubation, cells were washed with 10 ml (2 ml/107 cells) of depletion buffer at 400 x g for 10 minutes. The cell pellet formed was re-suspended in 250µl (50µl/10 cells) before magnetic separation was carried out. The MACS LD column (Miltenyi Biotech) was placed inside the magnetic groove of the magnet and primed by the addition of 2 ml of depletion buffer, with care being taken to avoid bubbles. The cell suspension was then added to the column carefully and allowed to pass through. This process could take some time depending on the number of CD45RO cells present in the sample. After all the cell suspension had passed through the column, 2 ml of depletion buffer was passed through the column. The collected CD45RO-T cells collected by this process were then re suspended in RPMI medium to a concentration of 4 x 10^6 cells/ml. Analysis of these cells after stimulation and intracellular cytokine staining was performed by FACS analysis.

5.3.4 Stimulation of Tr1 cells from freshly isolated tonsillar MNC and CD45RO+ depleted tonsillar MNC.

CD45RO-tonsillar MNC or freshly isolate tonsillar MNC were cultured for 3 days at 37°C with stimulation using a variety of different pneumococcal stimulants. After 3 days of culture, the frequency of Tr1 cells was measured using a combination of surface and intracellular staining. The co-staining of CD49b, LAG-3 and CD4 allowed for the identification of Tr1 cells and the intracellular staining of IL10 confirmed the cells were Tr1 cells.

5.3.4.1 Intracellular staining

Tr1 cells are a suppressive subset of regulatory T cell which exert their effect mainly though the production of IL10. Tr1 cells can be identified by the co expression of surface markers CD49b and Lag 3 and the high expression of IL10[1]. This is the combination of markers I was staining for in order to identify a cell population as being Tr1 cells.

To detect Tr1 cells, after incubation with BFA, adenotonsillar MNC were harvested from the plates into 1.5 ml Eppendorf tubes and washed with FACS staining buffer (0.02% PBS-BSA) by centrifugation at 600 x g for 8 minutes. After washing, 20 µl of Alexafluor 647 (BD Bioscience) conjugated CD49b, and 5 µl of PeCy7 (BD Bioscience) conjugated CD4 was added to each sample and tubes were incubated at 4 degrees for 30 minutes. Adenotonsillar MNC or PBMC were then washed using 1 ml of FACS staining buffer at 600 x g for 8 minutes. 350 µl of fixation/permeabilisation buffer was added to each sample and tubes incubated for 30 minutes at room temperature. Cells were then washed in permeabilisation buffer followed by centrifugation at 600 x g for 8 minutes, before the addition of 5 µl of APC (BD Bioscience) Conjugated IL10 and 20 µl of PE (R&D Systems) conjugated LAG3 to each sample. This was incubated at room temperature for a further 30 minutes followed by a final wash step using 1 ml of permeabilisation buffer per sample,

followed by centrifugation for 8 minutes at 600 x g. The final step was the addition of 500 µl of FACS staining buffer to each tube ready for FACS analysis.

5.4 Results

5.4.1 Gating strategy for the identification of Tr1 cells

For this study, Tr1 cells were defined as IL10-producing cells that were positive for both CD49b and LAG-3 when stained using intracellular cytokine staining. (Figure 5.4.1) The lymphocyte population was highlighted using the typical FSC and SSC configuration. Within the lymphocyte population the CD4+ cells were then defined in R2. R3 then highlights the cells within R2 which are both CD49b+ and LAG-3+ and are therefore defined as Tr1 cells. To confirm that these cells are Tr1 cells. The CD4+ population was also gated to show IL-10 positive cells and using this gate, the cells within the R3 region were confirmed to be IL-10 producing Tr1 cells

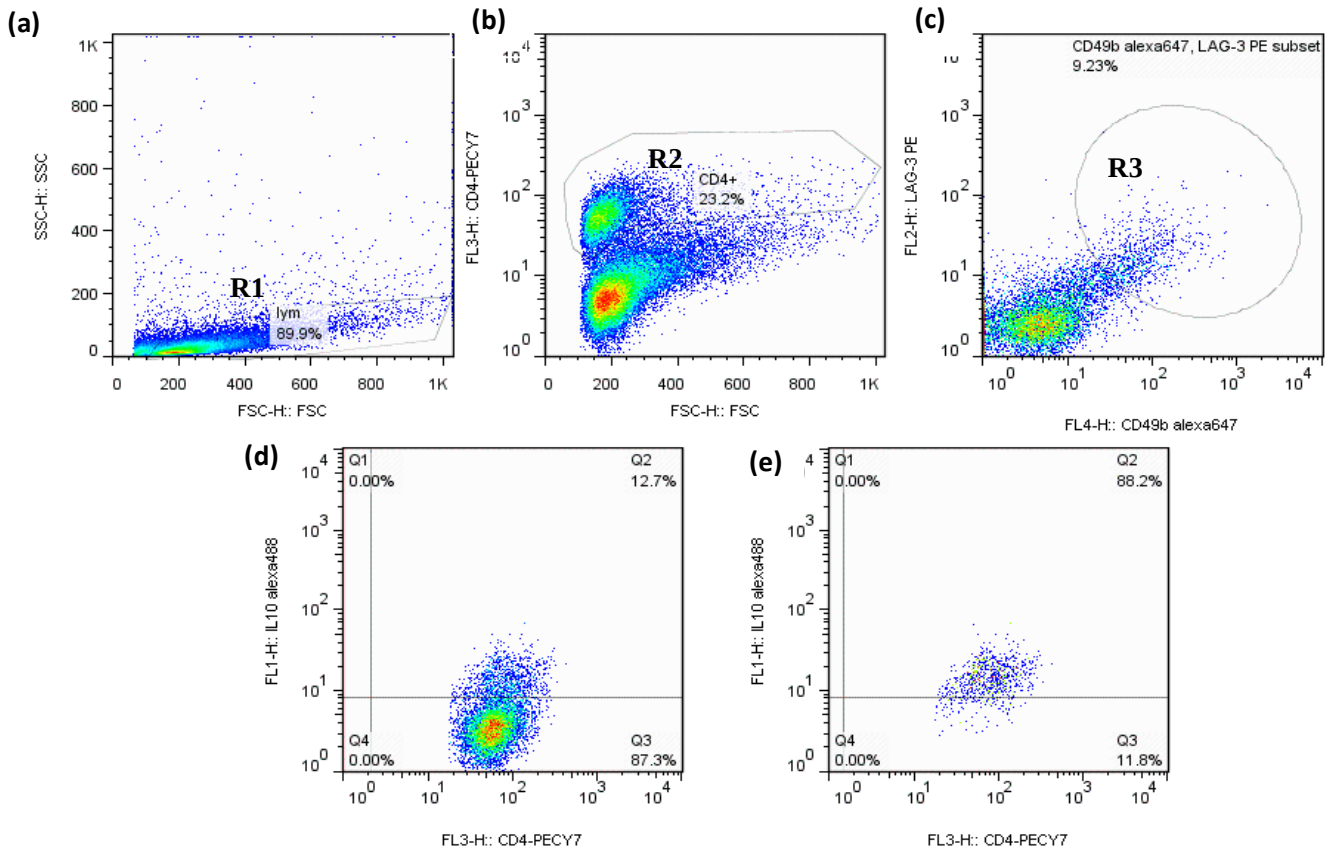


Figure 5.4.1 One representative figure that shows the gating strategy used for the identification of Tr1 cell frequency in freshly isolated tonsillar MNC

(a) The lymphocyte population is highlighted in R1, using the typical FSC and SSC configuration. (b) Within the lymphocyte population the CD4+ cells are defined in R2. (c) R3 then highlights the cells within R2 which are both CD49b+ and LAG-3+ and are therefore defined as Tr1 cells. (d) IL-10 positive cells within the CD4+ lymphocyte population. (e) In order to confirm the cells in R3 are Tr1 cells they are then gated against IL-10.

5.4.2 Optimal doses of pneumococcal CCS, pneumolysin toxoid W433F and purified capsular polysaccharide T3P and 6B to be used for the stimulation of Tr1 cells in human tonsillar MNC

The optimal doses of D39 WT pneumococcal CCS to use for the stimulation of Tr1 cells were determined prior to activation experiments (Figure 5.4.2a). For determining the optimal dosage for activation experiments the whole cell population (WCP) was used. Freshly isolated tonsillar MNCs were stimulated using a variety of doses of each D39 WT pneumococcal CCS to determine which caused the optimum level of activation. A total of 4

samples were stimulated (n=4) with a dose of 1, 2 and 4 µg/ml. The optimum dose for the activation of Tr1 cells was shown to be 1 µg/ml (Figure 5.4.2a). This concentration of 1 µg/ml was used in all experiments to stimulate tonsillar MNCs.

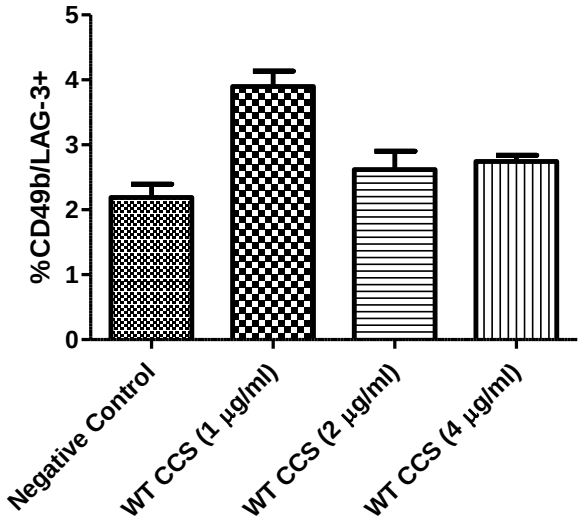


Figure 5.4.2a. Dose response curve of WT pneumococcal CCS for Tr1 cells activation.
Stimulation of tonsillar MNC with WT pneumococcal CCS shows the optimal concentration to be 1 µg/ml (n=4)

Concentration curves for W433F, T3P and 6B were done to determine the optimum dose for stimulation of freshly isolated tonsillar MNC for the activation of Tr1 cells. Figure 5.4.2b shows the dose curves for W433F (a), T3P (b) and 6B (c). The dose curve for pneumolysin toxoid W433F shows the optimal dose for stimulation is 10 ng/ml (n=2). For both T3P and 6B the optimal dose for stimulation is 1 µg/ml (n=2). These concentrations were used for the stimulation of human tonsillar MNC to see what effect they had on the activation of Tr1 cells.

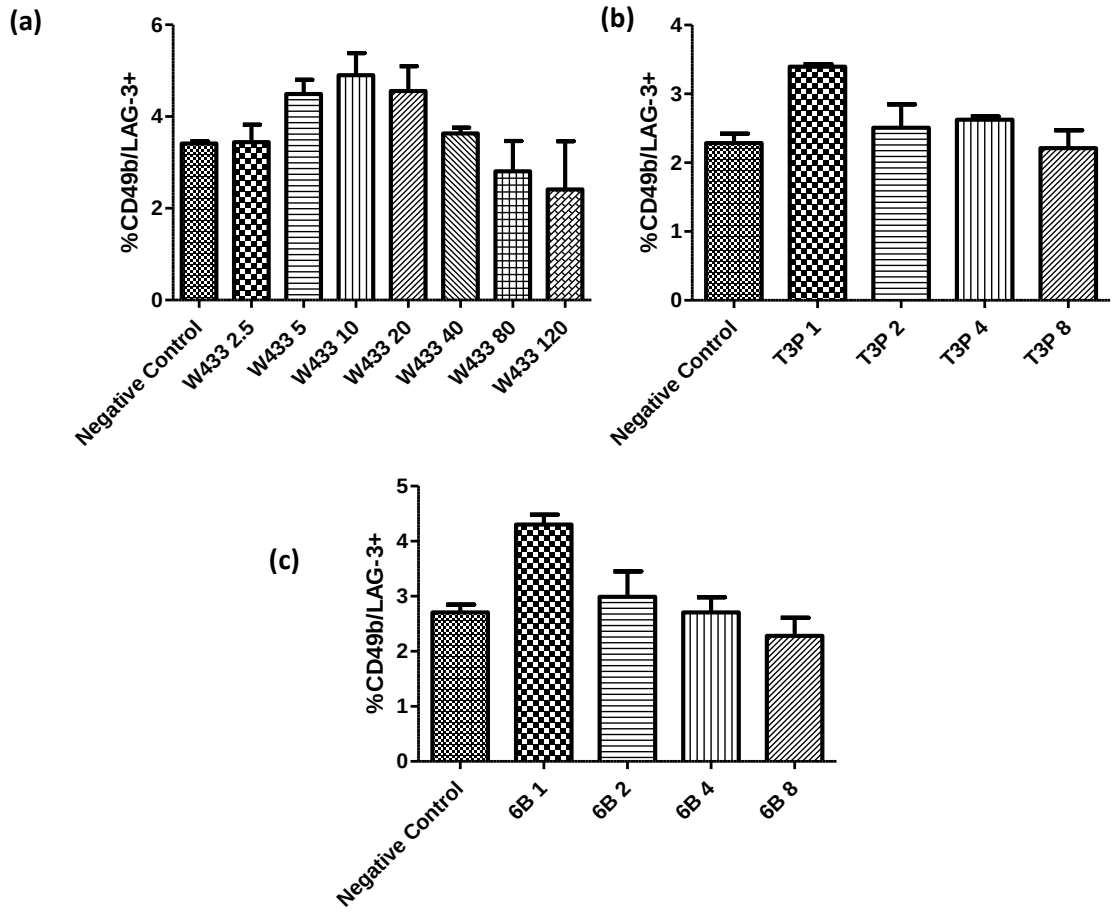


Figure 5.4.2b. Dose response curves for W433F, T3P and 6B for Tr1 cell stimulation

(a) The percentage of Tr1 cell activation by W433F is shown to be optimal at 10 ng/ml with higher concentrations seeming to have a toxic effect on the cells. **(b)** The frequency of Tr1 cell activation by T3P is shown to be optimal at 1µg/ml with higher concentrations seeming to have a toxic effect on the cells. **(c)** The frequency of Tr1 cell activation by 6B is shown to be optimal at 1µg/ml with higher concentrations seeming to have a toxic effect on the cells.

5.4.3 Stimulation of human tonsillar MNC with pneumococcal CCS deficient in Ply and capsular polysaccharide show lower frequencies of Tr1 cell activation compared to stimulation with WT pneumococcal CCS

Pneumococcal CCS was used to stimulate the WCP to determine if the absence of key pneumococcal virulence factors (pneumolysin and capsule) influence the activation of Tr1 cells. Pneumococcal CCS was used to stimulate freshly isolated tonsillar MNC at a concentration of 1 µg/ml as outlined in Figure 5.4.2. Figure 5.4.3 shows that D39 WT pneumococcal CCS stimulation of tonsillar MNC leads to the activation of 4.06% of Tr1 cells.

The use of Ply-/- CCS and Capsule -/- CCS for stimulation sees the percentage of Tr1 cell activation reduce significantly (2.37% and 2.46% respectively)

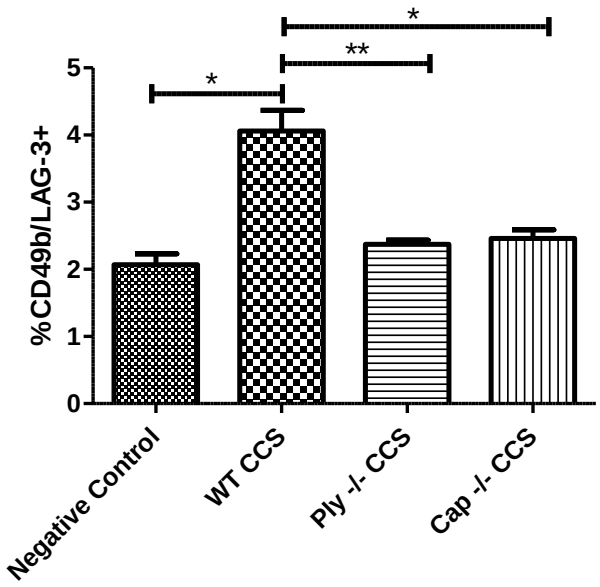


Figure 5.4.3. The frequency of Tr1 cell activation after stimulation with pneumococcal CCS
The frequency of Tr1 cells is shown to be significantly higher in tonsillar MNC upon stimulation with WT CCS compared to a negative control (2.07% for the negative control and 4.06% when stimulated with WT CCS). ($p = 0.0150$, $n = 4$). Ply -/- CCS shows significantly less activation compared to the WT CCS (2.37% compared to 4.06%) ($p = 0.0098$, $n = 4$). Cap -/- CCS shows significantly less activation compared to WT CCS (2.46% compared to 4.06%) ($p = 0.0242$ $n = 4$) Mean + SEM are shown *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.4.4 Stimulation of human tonsillar MNC with pneumolysin toxoid W433F and purified capsular polysaccharide (T3P and 6B) supports the importance of pneumolysin and polysaccharide in Tr1 cell activation

Data already shown (Figure 5.4.3) suggests that pneumococcal virulence factors pneumolysin and polysaccharide capsule may be important for the activation of Tr1 cells in human tonsillar MNC. To confirm this, human tonsillar MNC were stimulated using a toxoid of pneumolysin (W433F) and two types of purified capsular polysaccharide (T3P and 6B).

Data shown in Figure 5.4.4 shows that compared to a medium control with no stimulation in which the percentage of activated Tr1 cells is 5.43%, the addition of W433F elicited an increase in Tr1 cells to 8.16%. The stimulation of tonsillar MNC with both T3P and 6B also shows significantly more activated Tr1 cells (7.73% and 8.00% respectively) compared to the unstimulated medium control (5.43%). The stimulation of tonsillar MNC with a combination of the two virulence factors, W433F with T3P and W433F with 6B also shows significantly more Tr1 cell activation (8.65% and 7.16% respectively) compared to an unstimulated control (5.43%) but there is no significance when compared to just W433F, T3P or 6B alone.

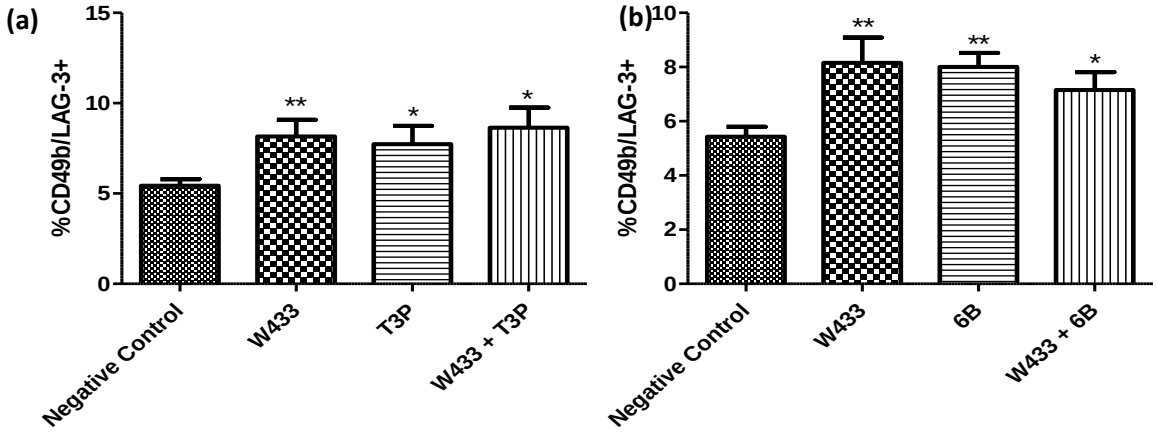


Figure 5.4.4 The frequency of Tr1 cell activation after stimulation with pneumococcal capsular polysaccharide (T3P and 6B) and toxoid of pneumolysin W433F

(a) The frequency of Tr1 cells after stimulation with purified pneumococcal capsular polysaccharide T3P is significantly higher when compared to an unstimulated negative control (7.73% compared to 5.43%, p = 0.0210, n = 7). The frequency of Tr1 cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (8.16% compared to 5.43%, p = 0.0089, n = 7). Stimulation using a combination of T3P and W433F shows a significant increase in Tr1 cell frequency compared to a negative control (8.65% compare to 5.43%, p = 0.0111, n = 7) but no significant difference compared to T3P and W433F alone. (**B**) The frequency of Tr1 cells after stimulation with purified pneumococcal capsular polysaccharide 6B is significantly higher when compared to an unstimulated negative control (8.00% compared to 5.43%, p = 0.0010, n = 7). The frequency of Tr1 cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (8.16% compared to 5.43%, p = 0.0089, n = 7). Stimulation using a combination of 6B and W433F shows a significant increase in Tr1cell frequency compared to a negative control (7.16% compare to 5.43%, p =0.0132, n = 7) but no significant difference compared to 6B and W433F alone. Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

5.4.5 Stimulation of CD45RO+ cell-depleted tonsillar MNC with pneumococcal CCS deficient in Ply and capsular polysaccharide show lower frequencies of Tr1 cell induction compared to stimulation with WT pneumococcal CCS

Freshly isolated human tonsillar MNC were depleted of CD45RO+ cells before being stimulated using pneumococcal CCS at concentrations previously described (Figure 5.4.2). WT pneumococcal CCS was used as well as pneumococcal CCS deficient in key pneumococcal virulence factors, pneumolysin and capsular polysaccharide to determine their importance in the induction of Tr1 cells. Figure 5.4.5 shows that when WT CCS is used to stimulate cells, the frequency of induced Tr1 cells is 4.11%. When pneumolysin deficient CCS is used, the percentage of induced Tr1 cells significantly reduces to 2.41%. The same thing is seen when Cap-/- CCS is used as the percentage if induced Tr1 cells is shown to be 2.48%.

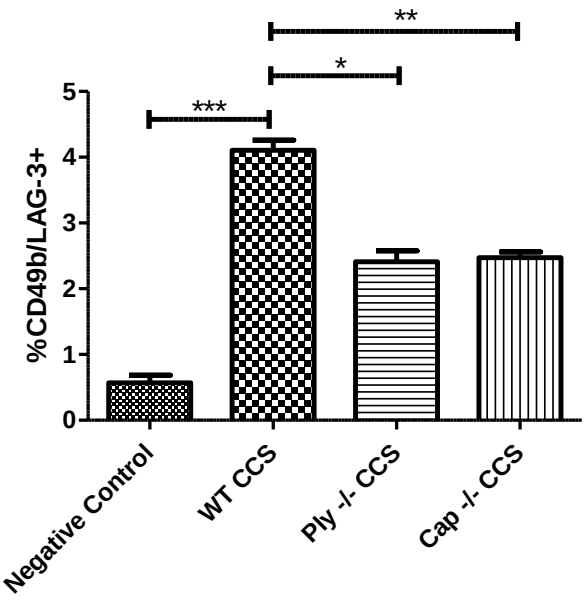


Figure 5.4.5. The frequency of Tr1 cell induction after stimulation with pneumococcal CCS
The frequency of Tr1 cells is shown to be significantly higher in tonsillar MNC upon stimulation with WT CCS compared to a negative control (0.57% for the negative control and 4.11% when stimulated with WT CCS). ($p = 0.0004$, $n = 4$). Ply -/- CCS shows significantly less activation compared to the WT CCS (2.41% compared to 4.11%) ($p = 0.0100$, $n = 4$). Cap -/- CCS shows significantly less activation compared to WT CCS (2.48% compared to 4.11%) ($p = 0.0050$ $n = 4$) Mean + SEM are shown *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.4.6 Stimulation of CD45RO+ cells-depleted human tonsillar MNC with pneumolysin toxoid W433F and purified capsular polysaccharide (T3P and 6B) supports the importance of pneumolysin and polysaccharide in Tr1 cell induction

Data already shown (Figure 5.4.5) suggests that pneumococcal virulence factors pneumolysin and capsular polysaccharide may be important for the induction of Tr1 cells from naïve CD4+ T cells in human tonsillar MNC. Freshly isolated human tonsillar MNC were depleted of CD45RO+ cells before they were stimulated using a toxoid of pneumolysin (W433F) and purified capsular polysaccharide (T3P and 6B) to confirm their important in Tr1 cell induction.

Data shown in Figure 5.4.6 shows that compared to a medium control with no stimulation (0.49%), the addition of W433F causes significantly more induction of Tr1 cells (3. 85%). The stimulation of tonsillar MNC with both T3P and 6B also shows significantly more activated Tr1 cells (4.24% and 4.23% respectively) compared to the unstimulated medium control (0.49%). The stimulation of tonsillar MNC with a combination of the two virulence factors, W433F with T3P and W433F with 6B also shows significantly more Tr1 cell activation (4.29% and 3.97% respectively) compared to an unstimulated control (2.36%) but there is no significance when compared to just W433F, T3P or 6B alone.

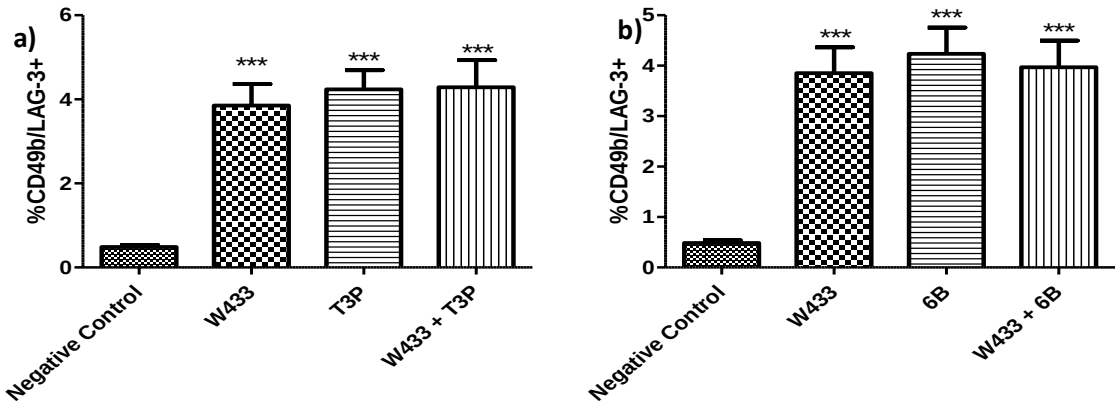


Figure 5.4.6. Figure 5.4.6. The frequency of Tr1 cell induction after stimulation with pneumococcal capsular polysaccharide (T3P and 6B) and toxoid of pneumolysin W433F

(a) The frequency of Tr1 cells after stimulation with purified pneumococcal capsular polysaccharide T3P is significantly higher when compared to an unstimulated negative control (4.24% compared to 0.49%, p = 0.0001, n = 7). The frequency of Tr1 cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (3.85% compared to 0.49%, p = 0.0004, n = 7). Stimulation using a combination of T3P and W433F shows a significant increase in Tr1 cell frequency compared to a negative control (4.29% compare to 0.49%, p = 0.0009, n = 7) but no significant difference compared to T3P and W433F alone. **(b)** The frequency of Tr1 cells after stimulation with purified pneumococcal capsular polysaccharide 6B is significantly higher when compared to an unstimulated negative control (4.23% compared to 0.49%, p = 0.0004, n = 7). The frequency of Tr1 cells after stimulation with W433F is significantly higher when compared to an unstimulated negative control (3.85% compared to 0.49%, p = 0.0004, n = 7). Stimulation using a combination of 6B and W433F shows a significant increase in Tr1 cell frequency compared to a negative control (3.97% compare to 0.49%, p = 0.0005, n = 7) but no significant difference compared to 6B and W433F alone. Mean + SEM are shown *p<0.05, **p<0.01, ***p<0.001.

5.5 Discussion

Colonisation of the nasopharynx by pneumococcus is known to be a pre-requisite for both invasive disease and for carriage and spread of the bacteria through the community. Understanding Interactions between pneumococcus and cells of the immune system is key to develop effective strategies to combat both infection and carriage. The role of regulatory T cells and their relationship with pneumococcus has been studied but little information is available about interactions in human nasopharynx which as the site of colonisation. In terms of regulatory T cells and pneumococcus, the best studied would be Foxp3+ Treg cells [90, 121, 275]. Previous studies have shown that pneumococcal whole cell antigen (WCA) is able to induce Foxp3+ Treg cells in the nasopharynx which suggests there are some components of pneumococcus that could be specifically involved in inducing this cell type[90]. Very little research has been done on other regulatory T cells such as type 1 regulatory T cells (Tr1). This chapter has looked at the relationship between Tr1 cells and pneumococcus and how specific pneumococcal components can both activate and induce Tr1 cells in tonsillar tissue.

Any potential vaccine candidate would interact with many different immune cells and it is therefore important to understand how any interactions affect the overall outcome of patient health.

Current pneumococcal vaccines have been successful in offering protection for those serotypes included but they do not confer protection for all serotypes and new serotypes have emerged to replace those that are now protected against[181 182]. The original capsular polysaccharide vaccines were effective in adults but had limited efficacy in young children as they were T cell independent. Moving on to polysaccharide conjugate vaccines in which the polysaccharide capsule was conjugated to protein, the vaccines became more immunogenic in young children and their effectiveness against serotypes included in the vaccine was very apparent. However, these vaccines are expensive to produce and limited

in serotype coverage. Pneumococcal vaccine research has therefore moved into looking at potential vaccines that target all pneumococcal serotypes regardless of their capsular polysaccharide. One way of doing this is through targeting a pneumococcal protein which is found in all serotypes. There have been several candidates suggested including pneumolysin[198].

The data presented in this chapter shows that in human tonsillar tissue, both pneumococcal capsular polysaccharide and pneumolysin can both activate and induce Tr1 cells. Section 5.4.3 shows a reduced response of Tr1 cells in tonsillar MNC following stimulation with pneumococcal CCS deficient in pneumolysin or capsule PS as compared to the wild type (WT) which has both. This suggest both pneumolysin and capsule could have a role to play in the activation of Tr1 cells.

The next step was to look at the effected of purified capsular polysaccharide (T3P and 6B) and a toxoid of pneumolysin (W433F) on the activation of Tr1 cells. Figure 5.4.4 shows that the pneumolysin toxoid W43F and both capsular polysaccharides used (T3P and 6B) were able to elicit a significant increase in Tr1 cell numbers compared to an unstimulated negative control. The combination of W433F and T3P or W433F and 6B didn't show any more significant activation than each component alone which is interesting as it seems to suggest they do the same thing without having an exacerbated effect on each other. It had previously been shown in a mouse model that the combination of T3P and Ply did confer an increased protective effect when compared to either the T3P or Ply alone[127].

This research looking at Tr1 cells responses to pneumococcal components is novel. There is already lots of research looking at Foxp3+ Treg cells responses but it is interesting to see that another category of regulatory T cells are reacting in much the same way. The stimulation using pneumolysin and capsular polysaccharide increased the numbers of Tr1 cells seen significantly which certainly seems to indicate their importance for Tr1 cell activation. The presence of pre-existing Tr1 cells could be due to previous colonisation

events by the pneumococcus in the nasopharynx. This would explain why stimulation saw the numbers of these cells increasing as fresh stimulation would activate cells that had previously encountered the stimuli.

We decided to deplete samples to see if Tr1 cells could be induced. In this instance we depleted samples of CD45RO+ cells. As with the activation experiments, initially cells were stimulated using pneumococcal CCS (Figure 5.4.5) to see whether any differences were observed in induction with and without pneumolysin and capsular polysaccharide. We did in fact once again see that when Ply-/- and Cap-/- CCS were used, the induction of Tr1 cells was significantly less than that when cells were stimulated using D39 WT pneumococcal CCS. This once again suggested pneumolysin and capsular polysaccharide are important players in Tr1 cell responses, so I then moved on to stimulation with purified pneumolysin toxoid (W433F) and purified capsular polysaccharide (T3P and 6B). (Figure 5.4.6) W433F stimulation alone showed significantly more Tr1 cells induction compared to the negative control. T3P and 6B stimulation alone also showed significantly more Tr2 cells induction compared to the negative control. And again, the combination of W433F with either of the two polysaccharides did not have an increased induction and was no different from to either components alone.

These results mirror those seen using pneumolysin and capsular polysaccharide for the activation of Foxp3+ Treg cells suggesting that several categories of regulatory cells are affected by pneumococcus. Capsular polysaccharide is currently used in pneumococcal vaccines with great success, although there is the issue with the expense and the limited coverage of serotypes. In this study, 2 different types of purified capsular polysaccharide were used to stimulate cells, T3P and 6B, and both were able to activate pre-existing Tr1 cells and induce Tr1 cells from naïve T cells. Pneumolysin was also able to both activate and induce Tr1 cells to much the same level as the capsular polysaccharide used. These results suggest Tr1 cells could have a role to play in the carriage of pneumococcus.

Regulatory T cells and their association with the mucosal immune system presents challenges to any therapies that would target bacteria that live at these sites. Any therapies would have to be non-toxic and not cause any worst effects through their modes of action. Regulatory T cells within the mucosal immune system are able to be induced upon stimulation by antigen, for example nasal antigen is able to induce Tr1 cells[276]. Tr1 cells have emerged as being very important in limiting excessive inflammatory responses[277, 278]. Their role during autoimmune diseases has been studied and they are regularly present during autoimmune inflammation[105, 279]. Regulatory T cells within mucosal tissue, whether its gut associated, nasopharynx associated or any other can be beneficial in preventing the excessive inflammation associated with unregulated effector cell responses[60]The results outlined here show that Tr1 cells are present in human tonsillar tissue and that pneumococcal antigen is able to activate pre exiting Tr1 cells and also induce Tr1 cells. The presence of Tr1 cells and their association with pneumococcal stimulation could suggest they have a role to play in the carriage of the bacteria. Their presence may allow the bacteria to persist within the nasopharynx without being cleared by effector CD4+ T cells which are being inhibited by their presence. Targeting any specific cell type such as Tr1 cells as a way of combatting carriage and therefore as a way of preventing spread of the bacteria through the population, is a challenging concept. To target these cells specifically would be very difficult and their reaction and responses to pneumococcal stimulation only give a small part of a much bigger picture of what interactions are occurring within the nasopharynx. But these cells do have some role to play in the carriage and persistence of infection and as such they could be worth exploring in more detail.

It is interesting to note that in both activation and induction experiments, the combined use of capsular polysaccharide and pneumolysin confers no additional protection compared to when either is used individually. It was thought that the stimulation with both components which are so effective at eliciting a response individually would perhaps cause

an additive effect on the response. However, in this instance no further benefits seem to be present when using both capsular polysaccharide and pneumolysin in combination. It could be argued that the effect of pneumolysin is not very relevant when current vaccines already use pneumococcal polysaccharide which has been shown to be just as effective as the pneumolysin in these experiments. However, the difference is that pneumococcal polysaccharide is so limited in the number of serotypes and pneumolysin is universally expressed, so perhaps a new vaccine that, moves away from, capsular polysaccharide and targets a protein is the way to move forward with pneumococcal vaccine research.

5.6 Summary

The results presented in this study suggest that specific capsular and protein components of pneumococcus may play a role in the induction and activation of Tr1 cells in human NALT. Data presented here suggests that both capsular polysaccharide and pneumolysin can both activate and induce Tr1 cells, and as such their role during pneumococcal carriage must be understood if a mucosal vaccine is to be used to protect against pneumococcal disease. Capsular polysaccharide is already used in pneumococcal vaccines but a vaccine that targets a universally conserved protein such as pneumolysin could offer protection against all serotypes of pneumococcus rather than just a few specific serotypes. More information is required to understand the exact role Tr1 cells and other regulatory and effector T cells play before a mucosal vaccine could be suggested but this data shows how Tr1 cells are affected by these specific pneumococcal components and provides some information which could feed into further vaccine research. What is clear is that Tr1 cells are present in the nasopharynx and are affected by stimulation with pneumococcal antigen and as such they could have a role to play in the carriage of pneumococcus.

Chapter 6

Role of TGF-β in the induction of Foxp3+ Treg cells in Human Nasal Associated Lymphoid Tissues

6.1 Introduction

Streptococcus pneumonia (pneumococcus) is an important human pathogen that is responsible for several invasive and non-invasive diseases which range in severity from mild otitis media to severe meningitis and pneumonia[2]. Pneumococcal disease is particularly prevalent in high risk groups including the elderly and young children and is a leading cause of childhood morbidity and mortality worldwide.

Foxp3+ Treg cells have an important role in the prevention of autoimmune disease and in the maintenance of immune tolerance. Foxp3+ Treg cells can be broadly classified into two subsets, naive Tregs (nTregs) and inducible Tregs (iTregs). iTregs are thought to acquire their suppressor activity through activation of CD4+CD25-FoxP3- T cells in the periphery upon stimulation by an antigen. Activation of iTregs occurs in the presence of TGF-β1[99]. It is thought that iTregs could have a particularly important role to play at mucosal surfaces where there is an abundance of TGF-β1. TGF-β1 is not required for the differentiation of nTregs but does seem important in promoting their survival in the periphery[99].

Foxp3+ Treg cells use the αβTCR for antigen recognition and are restricted by MHC-II molecules as with other CD4+ T cells[101]. It is thought that there are two mechanisms by which Treg cells exert their suppressive effects. The contact dependent method involves CTLA-4 on the Treg cell and CD80/86 on APCs[102]. CTLA-4 is expressed at high levels on CD4+CD25+ Treg cells and it is thought that the CTLA-4 interacts with the CD80/86 on APCs which leads to the activation of an enzyme called IDO (indoleamine 2,3 dioxygenase) in dendritic cells[280]. This ultimately leads to decreased activation of effector T cells[280, 281]. The second mechanism is mediated through immunosuppressive cytokines. Immunosuppressive cytokines are also important in Treg associated suppression. IL-10 and TGF-β released by Treg cells are responsible for down regulating MHC II and co-stimulatory

molecules on dendritic cells which affects their ability to present antigens and so stops the activation of CD4+ T cells[102].

Nasopharyngeal colonisation is very common in young children before natural immunity can develop. In the UK colonisation in children less than 3 years of age is between 40-50%[117] but in African children this can reach 90-100%[118, 119]. By the age of 3 most children have developed natural immunity against specific pneumococcal antigens. This natural immunity is responsible for the clearance of the pneumococcus from the nasopharynx before carriage status can be achieved. However, despite this natural immunity carriage can still occur throughout life and it is thought that Treg cells could have a role in this.

Due to the success of vaccines targeted to the polysaccharide capsule of the pneumococcus, it has classically been thought that protection against it was due to an antibody-based response[120]. However, more and more publications are now suggesting an important role for CD4+ T cell immunity in protection against the pneumococcus. MHC-II knockout mice which lack the ability to induce cell-mediated immunity through the presentation of antigen show prolonged carriage [120] suggesting CD4+ T cells have an important role to play in pneumococcal carriage rather than antibody-based responses.

In a recent study by Zhang et al, adenoidal cells from children who tested positive for pneumococcal carriage were found to contain higher numbers of Foxp3+ Treg cells when stimulated with pneumococcal whole cell antigen than those taken from children that tested negative[90]. The Treg cells present are of a memory phenotype suggesting they have been induced by a previous colonisation event. This association between carriage state and the increased number of Treg cells in the nasopharynx suggests that colonisation with the pneumococcus leads to the promotion of pneumococcus specific Treg cells. As well as this, the depletion of these cells before stimulation with whole cell antigen led to an increase in CD4+ T cell proliferation and the re-introduction of the Treg cells suppressed this effect[121].

This suggests that these memory Treg cells present in the nasopharynx have a potent inhibitory effect on CD4 T cell proliferation and may contribute to the persistence of carriage seen in young children.

The importance of regulatory T cell function in the nasopharynx and their role in the carriage of pneumococcus have made these cells an attractive study for looking at potential therapies against the bacteria. Understanding how Foxp3+ Treg cells are activated in the nasopharynx would inform new therapies for targeting the carriage of the bacteria.

6.1.1 Transforming growth factor beta (TGF-β)

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that is expressed in 3 different isoforms TGF-β1-3[206]. TGF-β is secreted by many different cell types has many roles including a role in regulating proliferation, differentiation, adhesion and migration[207]. After synthesis the TGFβ molecules are secreted as part of an inactive complex comprising of an N-terminal latency-associated peptide (LAP) and a mature TGF-β monomer at the C terminal. Homodimers of TGF-β and LAP interact in a non-covalent manner forming a complex known as small latent complex (SLC)[208]. LAP is then covalently linked to one of three latent TGFβ binding proteins (LGBTs) to form a larger complex called a large latent complex (LLC) which is then secreted out of the cell into the extracellular matrix[209].

The LLC will then remain in the extracellular matrix in an inactive state and requires further processing to release the active TGF-β product. For TGF-β to become active the LAP must be cleaved or modified in some way to expose the binding site of the TGF-β receptor[205].

6.1.2 Role of TGF-β in Foxp3+ Treg cell activation

TGF-β and Treg cells are both important in the control of immune responses and in the maintenance of immune homeostasis. TGF-β appears to play a role as an effector cytokine involved in the immunosuppressive function on Treg cells.

TGF-β 1 has a role in the induction of both inducible Tregs which have an inhibitory role on Th17 cells which are responsible for the production of numerous pro-inflammatory cytokines including IL17A[228].

There are several mouse models that have been established which allow the investigation of the role of TGF-β in immune regulation. TGF-β -/- mice allowed the realisation of how important TGF-β 1is in immune system regulation. The mice in this model develop a rapid wasting syndrome which leads rapidly to death[229]. Transgenic mice that have an impaired TGF-β signalling pathway show an increased susceptibility to the induction of disease, although it is not as severe as disease seen in TGF-β -/- mice[230]. Mice can survive for several months without developing severe disease. This can be explained by TGF-β acting on multiple cell types. So TGF-β can still influence some cells but not on others. So, mice show a susceptibility to the induction of allergic and autoimmune responses without developing spontaneous autoimmune disease.

6.1.3 αvβ8

αvβ8 is an integrin that has been shown to have a role to play in the activation of TGF-β[235]. Integrins are transmembrane receptors that act as a bridge for interactions between two cells or between cells and the extracellular matrix. Integrins are responsible for triggering chemical pathways which pass information about the exterior of the cell into the interior of the cell. In this way the chemical composition or the mechanical nature of the cell can be monitored, and events triggered at the cell surface can be rapidly responded to.

6.1.4 Role of αvβ8 and Foxp3+ Treg cells in TGF-β activation

TGF-β has been shown to play a role in Treg cell suppression of T cells but the mechanism by which this occurs isn't well understood. TGF-β is always produced in an inactive state and must first be activated before it can bind to its receptor and exert and effect[237]. Foxp3+

Treg cells have been shown to be able to activate TGF-β via the expression of the integrin αvβ8 and on activated Treg cells, this integrin is upregulated[235]. If Treg cells lack the expression of αvβ8 they become completely unable to suppress T cell mediated inflammatory responses[235].

6.2 Aims of study

In this chapter we have investigated:

1) The role of TGF-β in the activation of Foxp3+ Treg cells.
2) The role of the integrin avb8 in the activation of TGF-β from its latent state to its active state.

6.3 Experimental Design

To determine the role of TGFβ in the activation of Foxp3+ Treg cells, adenotonsillar MNC were stimulated using pneumococcal CCS for 72 hours. The supernatant was then collected and analysed by TGF-β ELISA to measure the concentration of TGF-β in culture for both cells that were unstimulated and cells that were stimulated with pneumococcal CCS. Cells were then pre-incubated with anti-TGFβ and anti- αvβ8 followed by stimulation for 72 hours with pneumococcal CCS to see if Foxp3+ Treg cell numbers were affected.

6.3.1 Patients and samples

Adenotonsillar tissue was obtained from both children and adults undergoing adenotonsillectomy. Full consent was obtained from each participating patient and full ethical approval for the study was given by the local ethics committee.

6.3.2 Pneumococcal CCS

Pneumococcal CCS was produced from a wild type strain of S. pneumoniae (D39) (NCTC7466)[238]. Bacterial frozen stocks were cultured overnight on blood agar plates (Fisher

Scientific, UK) (37°C, 5% CO2). After about 18 hours, typical α-haemolytic colonies of *S. pneumoniae* were observed. Several colonies were then used to inoculate Todd Hewitt Broth (THB) (Oxoid, UK) containing 5% yeast extract which was then cultured overnight (37°C, 5% CO2). The following day, the optical density of the culture was measured at 620nm and then this was checked every 30 minutes until exponential phase of growth was reached (OD of 0.4-0.5 at 620nm). The broth culture was centrifuged at 3000 x g for 30 minutes and the supernatant removed. The supernatant was then passed through a 0.45 µm filter followed by a 0.2 µm filter. Concentration of the pneumococcal culture supernatant was then achieved by centrifuging the sample at 3000 x g for 30 minutes in a Vivaspin15 concentrator (Sartorius Stedim Biotech, Germany). This process was repeated several times to ensure that the sample was concentrated tenfold. Samples were then aliquoted into 1.5 µl microcentrifuge tubes and stored at -80°C until use. The protein concentration of pneumococcal CCS was measured using the Bradford protein.

6.3.3 Cell culture and pre-incubation

Adenotonsillar MNC cultures were set up to measure *in vitro* TGF-β levels. Cells were stimulated using WT pneumococcal CCS, WT pneumococcal CCS pre-incubated with anti-TGF-β and WT pneumococcal CCS pre-incubated with anti-αvβ8. The process for the isolation of mononuclear cells from adenotonsillar tissue is outlined in the methods section. Briefly, adenotonsillar tissue was washed before being transferred to a sterile petri dish containing HBSS for mashing of the sample to allow the release of cells. The media containing the cells was passed through a filter before being layered carefully on to ficoll and centrifuged to allow the separation of the lymphocytes. The lymphocytes were then washed, and the pellet re-suspended before being counted using an automatic cell counter. The cells were then re-suspended to a working concentration of 4 x 10^6. To see the effect of TGF-β on Foxp3+ Treg cell activation some cells were added to an eppendorf with anti-TGFβ

and incubated at 37°C for 30 minutes before being cultured. To see if αvβ8 had any effect of Foxp3+ Treg cell activation some cells were added to an eppendorf and pre-incubated at 37°C for 30 minutes with anti-αvβ8. A sample of cells was added to a plate with no additional stimulation as a negative control. Cells were added to the plate with no pre-incubation and cells pre-incubated with anti-TGFβ and anti-αvβ8 were added. These cells were then stimulated with WT pneumococcal CCS before the plate was incubated at 37°C for 72 hours and supernatant was collected and stored in the freezer until use.

6.3.4 Intracellular cytokine staining of Foxp3

To determine the effects of αvβ8 and TGF-β on the activation of Foxp3+ Treg cells on tonsillar MNC, cells were stimulated with WT pneumococcal CCS both before and after pre-incubation with anti- αvβ8 or anti-TGF-β. This meant that the levels of Foxp3+ Treg cell activation could be measured by intracellular cytokine staining to see if any differences were observed. The detailed method for the staining of Foxp3+ Treg cells can be found in chapter 2. Briefly, tonsillar MNC were isolated from tonsillar samples by ficoll gradient centrifugation. Cells were cultured in 96 well flat bottom plates (corning) at a concentration of 4×10^6 in RPMI (sigma) supplemented with glutamine, streptomycin, penicillin and 10% FBS. Cells were stimulated with WT pneumococcal CCS, both before or after pre-incubation with anti-αvβ8 or anti-TGF-β. Cells were then harvested into Eppendorf tubes before being stained with CD4 and Foxp3 following the method outlined in chapter 2.

6.3.5 TGF-β ELISA

Adenotonsillar MNC were stimulated using WT pneumococcal CCS, WT pneumococcal CCS pre-incubated with anti-TGF-β and WT pneumococcal CCS pre-incubated with anti-αvβ8. Supernatant was collected after 72 hours before analysis using TGF-β ELISA. The method is outlined in methods section. Briefly, capture antibody was used to coat 96 well plate and left overnight at 4°C. The plate was washed before being blocked and incubated for 1 hour

at room temperature. Standards and samples were prepared and added to the plate after washing before the plate was incubated for 2 hours at room temperature. The plate was washed, and detection antibody was added before the plate was incubated at room temperature for 1 hour. Avidin-HRP was added to each well for 30 minutes at room temperature before the plate was washed and substrate solution was added. The plate was left for 15 minutes in the dark before the addition of stop solution. Finally, the pate was read at 450 nm and the concentration of TGF-β measured against the standard curve.

6.4 Results

6.4.1 TGF-β present in medium control but level increases when stimulated with WT pneumococcal CCS

Freshly isolated tonsillar MNC were stimulated for a period of 72 hours using WT pneumococcal CCS before the supernatant was collected for analysis. The in vitro production of TGF-β in tonsillar MNC after stimulation was then measured using the TGF-β ELISA assay. After stimulation using D39 WT pneumococcal CCS, the level of TGF-β in the culture supernatant was found to be significantly higher than in a medium control which received no stimulation (Figure 6.4.1)

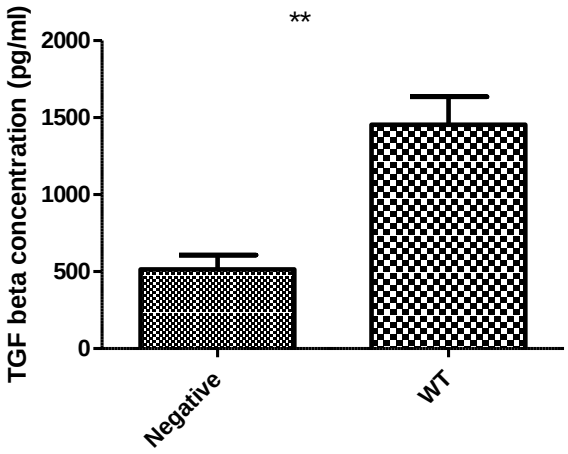


Figure 6.4.1. TGF-β production in tonsillar MNC following stimulation by pneumococcal CCS
The level of the cytokine TGF-β was measured by ELISA assay after 72 hours both with and without stimulation using WT pneumococcal CCS. The TGF-β concentration was significantly higher in the samples stimulated with WT pneumococcal CCS (1453.4 pg/ml) compared to the negative control samples (513.4 pg/ml) Mean+SEM are shown**p<0.01,

6.4.2 Foxp3+ Treg cell percentage increases upon stimulation of tonsillar MNC with WT pneumococcal CCS

Freshly isolated tonsillar MNC were cultured for 3 days both with and without stimulation using WT pneumococcal CCS. The stimulation of these cells with the pneumococcal CCS caused the percentage of Foxp3+ Treg cells to increase significantly. Figure 6.4.2 shows the gating strategy used to identify Foxp3+ Treg cells both with and without stimulation using pneumococcal CCS and the percentage of Foxp3+ Treg cells identified under both conditions.

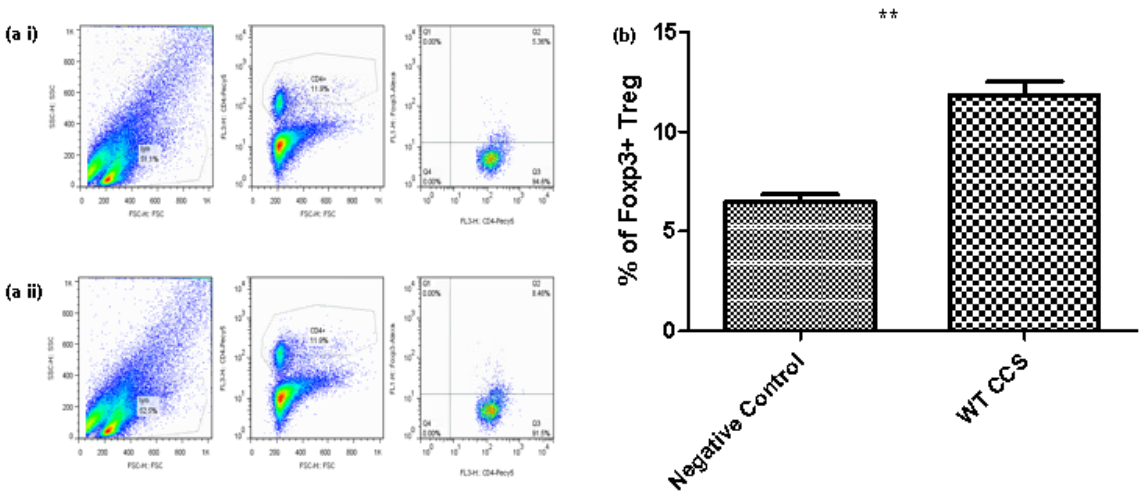


Figure 6.4.2. Foxp3+ Treg cell percentage in tonsillar MNC following stimulation by WT pneumococcal CCS

The percentage of Foxp3+ Treg cells were by intracellular cytokine staining after 72 hours of stimulation both with and without stimulation using WT pneumococcal CCS. **(a i)** Tonsillar MNC sample with no stimulation **(a ii)** Tonsillar MNC sample after stimulation using WT pneumococcal CCS. R1 highlights the lymphocyte population, defined using typical FSC and SSC. Within the lymphocyte population the CD4+ cells are then defined in R2. Finally, the Foxp3+ Treg cells are identified by the gating of the CD4+ population (R2) and the positive staining of Foxp3 **(b)** The percentage of Foxp3+ Treg cells was significantly higher in the samples stimulated with WT pneumococcal CCS (11.82%) compared to the negative control samples (6.47%) ($p=0.0076$, $n=6$) Mean+SEM are shown**$p<0.01$.

6.4.3 TGF-β level decreases upon pre-incubation using anti-TGF-β or anti-αvβ8

Freshly isolated tonsillar MNC were pre-incubated with anti TGF-β or anti αvβ8. Cells were then stimulated using WT pneumococcal CCS and incubated for 3 days. The in vitro production of TGF-β in tonsillar MNC after stimulation was then measured using the TGF-β ELISA assay. Cells pre-incubated with anti TGF-β showed significantly decreased TGF-β production compared to a negative control. Cells pre-incubated with anti-αvβ8 showed significantly decreased TGF-β production compared to a negative control (Figure 6.4.3).

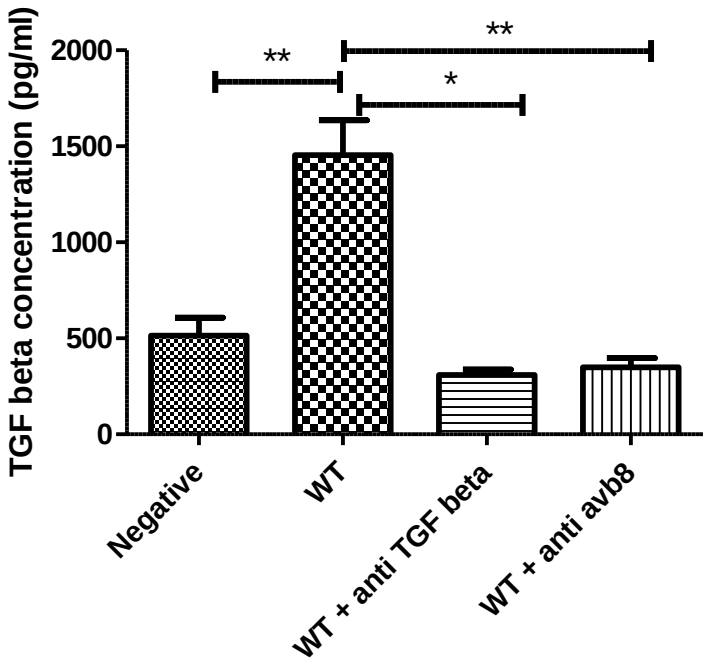


Figure 6.4.3. TGF-β production in tonsillar MNC following stimulation by pneumococcal CCS after pre-incubation with anti-TGF-β or anti-αvβ8

The concentration of TGF-β is shown to be significantly lower in cells stimulated with WT pneumococcal CCS that were pre-incubated with anti TGF-β (308.548 pg/ml) when compared to cells stimulated with WT pneumococcal CCS alone. (1453.41 pg/ml) (p = 0.0056, n = 4) The concentration of TGF-β is shown to be significantly lower in cells stimulated with WT pneumococcal CCS that were pre-incubated with anti αvβ8 (348.627 pg/ml) when compared to cells stimulated with WT pneumococcal CCS alone. (1453.41 pg/ml) (p = 0.0123, n = 4) Mean + SEM are shown *p<0.05, **p<0.01,

6.4.4 Foxp3+ Treg cell percentage is reduced when cells are pre-incubated with anti- αvβ8 or anti-TGF-β

Freshly isolated tonsillar MNC were pre-incubated with anti-TGF-β or anti-αvβ8. Cells were then stimulated using WT pneumococcal CCS and incubated for 3 days. To confirm that the TGF-β levels observed from the ELISA results changed as a result of Foxp3+ Treg cell activation, the percentage of Foxp3+ Treg cells were measured by intracellular cytokine staining followed by FACS analysis. Figure 6.4.4 shows the percentage of Foxp3+ Treg cells identified and a representative example of the gating strategy used to isolate Foxp3+ Treg cells from other cells.

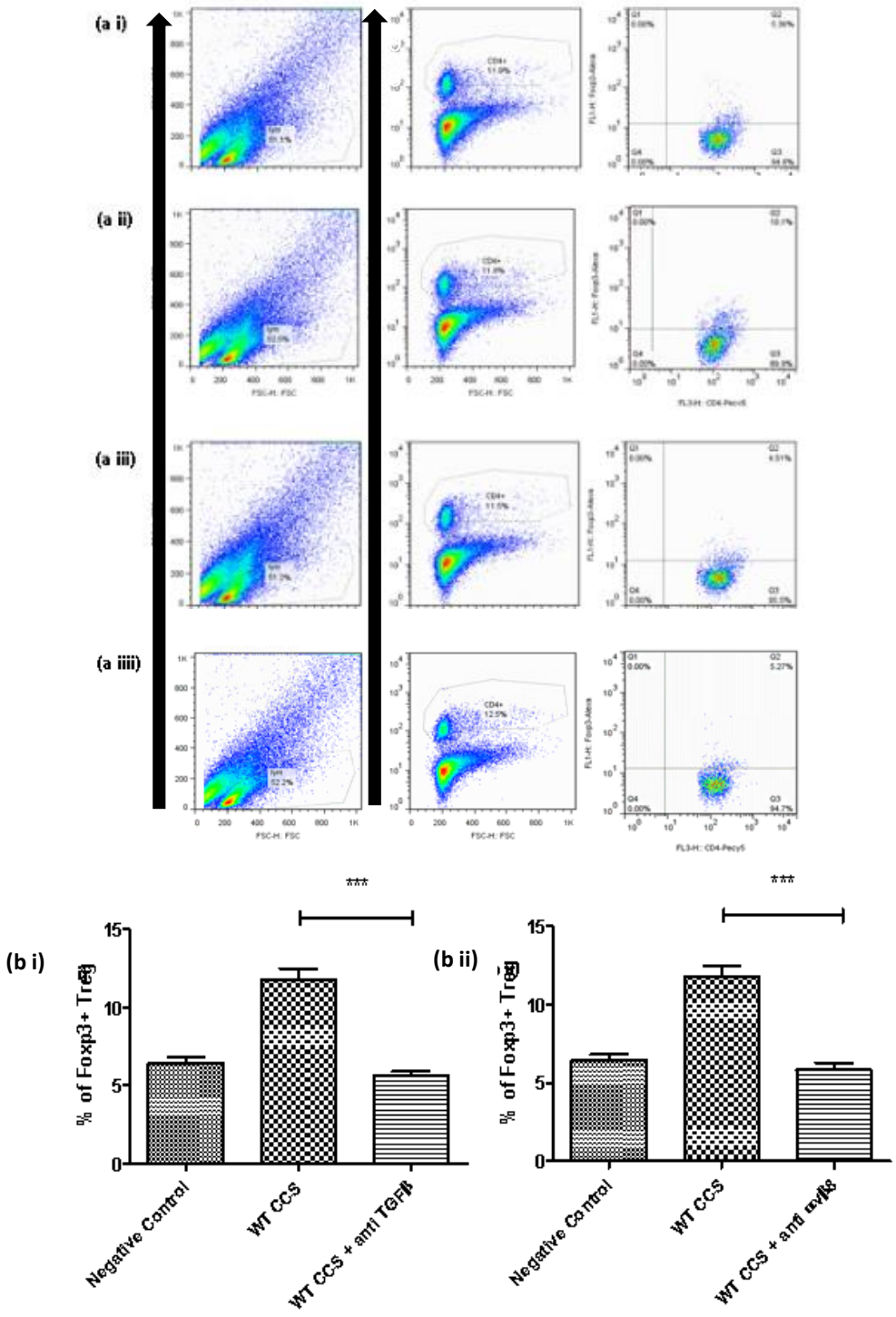


Figure 6.4.4. Foxp3+ Treg cell percentage in tonsillar MNC following stimulation by pneumococcal CCS after pre-incubation with anti-TGF-β or anti-αvβ8

(a i) Tonsillar MNC sample with no stimulation **(a ii)** Tonsillar MNC sample after stimulation using WT pneumococcal CCS. **(a iii)** Tonsillar MNC sample after stimulation with WT pneumococcal CCS when cells were pre incubated with anti-TGF β, **(a iiii)** Tonsillar MNC sample after stimulation with WT pneumococcal CCS when cells were pre incubate with αvβ8. R1 highlights the lymphocyte population, defined using typical FSC and SSC. Within the lymphocyte population the CD4+ cells are then defined in R2. Finally, the Foxp3+ Treg cells are identified by the gating of the CD4+ population (R2) and the positive staining of Foxp3 **(b i)**The percentage of Foxp3+ Treg cells is shown to be significantly lower in cells stimulated with WT pneumococcal CCS that were pre-incubated with anti TGF-β (5.65%) when compared to cells stimulated with WT pneumococcal CCS alone. (11.82%) ($p = 0.0003$, $n = 6$) **(b ii)** The percentage of Foxp3+ Treg cells is shown to be significantly lower in cells stimulated with WT pneumococcal CCS that were pre-incubated with anti αvβ8 (5.82%) when compared to cells stimulated with WT pneumococcal CCS alone. (11.82%) ($p = 0.0005$, $n = 6$) Mean + SEM are shown, ***$p<0.001$.

6.5 Discussion

CD4+ T cells have now been established as being an important part of protective immunity against pneumococcus, with MHC class-II deficient mice which have a significantly reduced number of CD4+ T cells being incredibly susceptible to infection by pneumococcus[262]. The role of pro-inflammatory CD4+ T cells during pneumococcal infection has been studied in detail[282, 283] but the role of regulatory cells hasn't been studied in as much detail and as such is an important gap in our knowledge about the immunology associated with pneumococcal carriage and disease. TGF-β is thought to be important in the differentiation of naïve CD4+ T cells into Foxp3+ Treg cells which can then proliferate and lead to suppression of the immune system[284, 285]. A study by Liao et al, showed that SPY1, a live attenuated vaccine against pneumococcus, is able to induce protective Treg cells[286] which are vital for the maintenance of homeostasis of the immune system and prevent infection associated inflammation[287]. In addition to this they showed that the addition of a peptide, P17[286, 288], which inhibits TGF-β and therefore reduces the numbers of Foxp3+ Treg cells, impairs the effectiveness of SPY1 which suggests the ability of SPY1 to activate TGF-β and activate Treg cells is important for producing protective Treg immune responses in the nasopharynx[287]. This suggests TGF-β has an important role to play in the activation of Foxp3+ Treg cells and the carriage of pneumococcus in the nasopharynx. This protective role of regulatory T cells has also been shown in a mouse study by Neill et al[275]. This study showed that regulatory T cells have an important role to play in the prevention of immune mediated damage in the lungs of infected mice and that TGF-β has an important role to play in this[275]. They used 2 mice models which react very differently to pneumococcal infection[289] and infected the lungs with pneumococcus. CBA/Ca mice which have fewer T regulatory cells and therefore less TGF-β showed insufficient immunosuppression and the bacteria was able to disseminate into the bloodstream before they could be cleared. In contrast, in BALB/c mice, a controlled and regulated pro-inflammatory environment was

created quickly after infection and bacteria were cleared. The presence of Treg cells in this environment acted to limit pro inflammatory responses to prevent tissue damage and maintain the integrity of the lungs and prevent bacteria getting into the bloodstream[275].

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that is expressed in 3 different isoforms TGF-β 1-3[206]. TGF-β is secreted by many different cell types has many roles including a role in regulating proliferation, differentiation, adhesion and migration[207]. TGF-β molecules are proposed to act as cellular switches that regulate processes such as immune function. Many cell types have TGF-β receptors and as such can be either positively or negatively regulated by TGF-β[207]. TGF-β exists in an inactive or latent form and must be activated to be able to exert its suppressive effect[205]. The TGF-β molecules are secreted as part of an inactive complex in its latent form. Latent TGF-β is complexed with an N-terminal latency-associated peptide (LAP) and a mature TGF-β monomer at the C terminal[205]. This complex is known as the large latent complex (LLC) which is then secreted out of the cell into the extracellular matrix[209].

The LLC will then remain in the extracellular matrix in an inactive state and requires further processing to release the active TGF-β product[210]. The presence of TGF-β in an inactive complex allow for the closer regulation of TGF-β signalling, as many cells express TGF-β receptors, but it is not always beneficial for TGF-β to be exerting an effect. TGF-β present in its latent state allows it to quickly be cleaved to its active state under the right conditions. The ubiquitous expression of TGF-β and its receptor suggest that its regulation must be complex and multifactorial but one of the most important mechanisms of preventing the effect of TGF-β is by stopping the cleavage of the LLC to release active TGF-β.

TGF-β synthesis is widespread as is expression of its receptors, but its activity is limited to areas where it is released from its latent state into its active state.

Foxp3+ Treg cells are important for the regulation of effector T cell responses which, if allowed to go unchecked, could cause severe host inflammation with damage to cells and tissues[228]. TGF-β is secreted by many different cell types including T cells macrophages and dendritic cells[228]. Its key functions include regulation of inflammatory cell responses and T cell regulation and differentiation. TGF-β has been shown to play a role in differentiation of both regulatory T cells and effector Th17 cells so is able to influence both regulatory responses as well as effector pro-inflammatory responses[228].

My results show that when freshly isolated tonsillar MNC are stimulated using WT pneumococcal CCS the concentration of TGF-β is increased compared to a negative control with no stimulation (Figure 6.4.1). To determine whether it was Foxp3+ Treg cells that were changing the levels of TGF-β, tonsillar MNC both with and without stimulation using WT pneumococcal CCS were analysed by intracellular cytokine staining and FACS analysis to see how the percentage of Foxp3+ Treg cells changed (Figure 6.4.2).

My results suggest that TGF-β has a role to play in the activation of Foxp3+ Treg cells and that the integrin αvβ8 may be important for the activation of TGF-β from its latent to active state. Stimulation of freshly isolated tonsillar MNCs using WT pneumococcal CCS shows a significantly higher concentration of TGF-β compared to a negative control. When the cells are first pre-incubated with anti-TGF-β the concentration of TGF-β reduces significantly. Anti-TGF-β neutralises the effect of TGF-β and this suggests the TGF-β in the cell supernatant has a critical role in Foxp3+ Treg cell induction.

The next stage in the story was looking at possible mechanisms for TGF-β activation as when it is secreted from cells it is in its inactive latent state. Before the latent TGF-β can exert an effect, it must first be cleaved into its active state. It has been suggested that the integrin αvβ8 could have a role to play in the activation of TGF-β. Freshly isolated tonsillar MNC were stimulated using pneumococcal WT CCS, both alone and that had been pre-

incubated with anti- TGF-β and anti-αvβ8. The supernatant was harvested and analysed by ELISA to see how the concentration of TGF-β was affected (Figure 6.4.3). Cells that were stimulated with WT Pneumococcal CCS alone showed a concentration of (11.82%). When cells were pre-incubated with anti- TGF-β or ant avb8 the concentration of TGF-β was significantly reduced to 5.65% and 5.82% respectively. Once again, In order to determine whether it was Foxp3+ Treg cells that were changing the levels of TGF-β, tonsillar MNC both with and without stimulation using WT pneumococcal CCS were analysed by intracellular cytokine staining and FACS analysis to see how the percentage of Foxp3+ Treg cells changed (Figure 6.4.4).

Knowing the mechanism through which Foxp3+ Treg cells exert their suppressive effects would allow for a more informed therapeutic strategy against pneumococcal infection. The nasopharynx has long been established as the site of colonisation of pneumococcus with more colonisation events occurring in the early years of life[90] when infection can be more severe.

A protective role of regulatory cells in pneumococcal disease and infection has been shown in several studies[275, 287] as has already been mentioned. The fact that regulatory T cells are clearly playing such an important role in pneumococcal disease and carriage just highlights how important it is for us to understand the immunology of pneumococcal infection, both of effector and regulatory T cells. A clear and complete picture is needed to be able to create an effective therapeutic strategy against infection. Vaccination has proven to be an effective strategy against pneumococcus and the evolution of pneumococcal vaccines from polysaccharide vaccines to conjugate vaccines has shown effective at reducing the instances of colonisation an invasive disease[29, 178, 187 188, 189]. Current pneumococcal vaccines can be administered by injection or mucosally but are mainly delivered via injection[287]. A mucosal vaccine which is administered directly into the nose could prove to be an effective

way of stimulating both a systemic immune response and a mucosal immune responses whereas an injected vaccine stimulates predominantly the systemic immune system and therefore less efficient in affect the carriage and clearance if the bacteria from the nasopharynx [287].

The results here all underlines the importance of knowing what regulatory immune responses are elicited by pneumococcus and how they are activated. It is clear that Treg play an important role during pneumococcal colonisation and disease and understanding these cell responses could be important for the future therapeutic strategy against pneumococcal colonisation and disease.

6.6 Summary

The results presented in this study suggest that the TGF-β has a role to play in the activation of Foxp3+ Treg cells. Given that TGF-β is present in its latent, inactive state and must be activated to be able to exert its inhibitory effects, we investigated the possible role of the integrin αvβ8 in the activation of TGF-β. Results presented here suggest that αvβ8 could have a role to play in TGF-β activation. Understanding the activation of these cells and how they are activated is important for understanding why they are present during pneumococcal carriage and what role they have to play during persistence of infection.

Chapter 7

Discussion and conclusion

7.1 Discussion

Streptococcus pneumoniae (pneumococcus) infection remains a global problem and is a major cause of morbidity and mortality worldwide, being responsible for many both invasive and non-invasive diseases such as pneumoniae, meningitis and bacteraemia[40]. Pneumococcus is particularly prevalent in developing countries and mainly affects those in at risk groups such as the very young, the elderly and those with underlying health issues[290]. Each year pneumococcal diseases cause the deaths of approximately 1 million children under 5 years of age[25].

Although pneumococcus can cause serious illness and complications in at risk individuals, many people are harmless carriers of the bacteria. The nasopharynx is the natural site of pneumococcal colonisation and many individuals can be colonised asymptomatically without becoming seriously ill[3]. These people then become carriers for the spread of the bacterium through the community. Young children are very common carriers of the bacteria and in some populations as many as 90% of children under 2 are colonised[4]. The diseases caused by pneumococcus occur when the bacteria is able to spread from the nasopharynx into other areas of the body, areas which at other times would remain sterile such as the lungs, brain or blood[4].

CD4+ T cells have now been established as being an important part of protective immunity against pneumococcus, with MHC class-II deficient mice which have a significantly reduced number of CD4+ T cells being incredibly susceptible to infection by pneumococcus[262]. The role of pro-inflammatory CD4+ T cells during pneumococcal infection has been studied in detail[282, 283] but the role of regulatory T cells has not been studied in as much detail and as such is an important gap in our knowledge about the immunology associated with pneumococcal carriage and disease.

It has been hypothesised that mucosal regulatory T cells in NALT may have a role to play in mediating pneumococcal carriage in the nasopharynx. In this study, we investigated the association of human mucosal regulatory T cells Foxp3+ Treg and Tr1 cells with pneumococcal antigens by analysing the numbers of Foxp3+ Treg and Tr1 cells in children and adult tonsillar tissue.

The nasopharynx is the site of colonisation of pneumococcus and colonisation is a common event in young children. Colonisation of the nasopharynx by pneumococcus is a pre-requisite for disease and can occur asymptomatically with no disease being present at all. Colonisation is also responsible for the spread of the bacteria through the community. This suggest that the reduction of carriage through vaccination could be an effective means of preventing pneumococcal disease.

Both adult and children tonsillar tissue was shown to have significantly more Tr1 and Foxp3+ regulatory T cells compared to PBMC samples. (Figure 3.4.1b, Figure 3.4.1c, Figure 3.4.2b, Figure 3.4.2c) This suggests that the mucosal compartment in the nasopharynx is exposed to increased numbers of microbes compared to the peripheral blood which experiences very little microbial exposure. The systemic compartment is a usually sterile site, whereas the nasopharynx is a site of microbial colonisation, so it is consistent with the hypothesis that an increased exposure to microbes in the nasopharynx leads to an increase in T regulatory cells.

We have also shown that the frequency of both Foxp3+ Treg cells in tonsillar MNC and PBMC changes with age. Children tonsillar samples were shown to have significantly more Tr1 and Foxp3+ regulatory T cell than adult samples. (Figure 3.4.3, Figure 3.4.4). Presumably this is due to the tonsils being the site of colonisation and children experiencing high numbers of colonisation event compared to adults. It is thought that

almost all children are colonised by at least one pneumococcal serotype in their first couple of years of life.[5]

One of the main age groups targeted by vaccines, including pneumococcal vaccines in those under the age of two. The reasons for this are quite clear, babies are born and move from a sterile environment and are instantly bombarded by a whole host of new and potentially dangerous pathogens, all the while with an immune system that isn't as developed as it will become[291]. An infant has no antigen experience and as such have to rely on their innate immune system to protect them from harmful antien[292, 293]. Pneumococcal polysaccharide vaccines produced a poor immunogenic response in this age group due to the limitations of the infant's immune system, an issue which has been overcome by the development of pneumococcal polysaccharide conjugate vaccines[172]. As we age and become exposed to more pathogens, our immune system develops and strengthens, and we are more able to defend ourselves from infection. A memory response is formed which is triggered upon encounter with the same antigen, mounting a much quicker and more effective response to get rid of the pathogen compared to an antigen that is encountered for the first time[51].

A strong memory T cell presence is seen in young children, which declines with age. Babies do not have the ability to launch a successful immune response against pathogens and the presence of memory T cells could be a mechanism with which they prevent and regulate excessive inflammatory responses[294]. This could be one of the reasons why vaccine induced responses are so poor from infants, the presence of Treg cells dampens down the pro inflammatory responses. Treg cells are induced by pathogens to regulate inflammatory responses so it makes a lot of sense that a vaccine would also influence these cells as they play a large part on immune responses against pathogens. It would be logical to assume that the Treg cell responses would be beneficial, preventing an excessive pro inflammatory immune response that could cause damage to the host such as tissue damage[291]. However,

studies have shown that the presence of Treg cells can interfere with vaccine induced immunity to the extent that the effects of the vaccine hampered which has led to studies in which Treg cells are depleted prior to vaccination which leads to a more successful vaccine induced immune response[295-297].

There is already research looking at Foxp3+ Treg cell numbers in the nasopharynx and peripheral blood but to the best of my knowledge this is the first research to look at Tr1 cells numbers in tonsillar samples of healthy adults and children and to compare Tr1 cell frequencies in 2 separate compartments, the NALT and PBMCs. The pattern of results observed is the same as that seen with Foxp3+ Treg cells, with much higher frequencies observed in the nasopharynx, where colonisation occurs, compared to the peripheral blood which is a sterile site that doesn't encounter pathogenic stimulation. This could suggest that Tr1 cells are also activated or primed by colonisation events by pneumococcus. Tr1 cells could play a similar role in inhibiting the clearance of the bacteria from the nasopharynx and could play a role in the spread of the bacteria through persistence of carriage.

The nasopharynx is the site of colonisation for pneumococcus, where it can be a harmless commensal bacterium. However, the colonisation without clearance of the bacteria allow it to spread through communities and if it is able to migrate from the nasopharynx into other site such as the lungs or blood it can cause severe disease[4]. Due to the protective effects of pneumococcal polysaccharide-based vaccines, antibodies against the capsule have traditionally been thought to be the main component of natural immunity against pneumococcus[298]. More recently, CD4+ T cell responses against pneumococcus have increasingly become recognised as being an important player in immune responses against the bacteria, both in promoting clearance of bacteria by inducing Th17 mediated pro-

inflammatory responses, but also in carriage of the bacteria through the activation of regulatory T cells responses[120].

It has been shown that pneumococcal antigen specific Th17 cells present in mucosal tissue increase with age. As we age we are exposed to increasing numbers of colonisation events and this could be why the numbers of effector CD4+ T cells increase with age[299]. The colonisation and carriage of pneumococcus is assumed to be regulated by natural immunity to pneumococcus which begins to be acquired from very early infancy[300]. With increased exposure events, the build-up of immunity against pneumococcus is thought to be the cause of the gradual decrease in pneumococcal carriage and infection which is seen with age[301, 302]. Despite this, some children experience persistence of carriage and understanding how this works is essential to being able to develop better ways of fighting pneumococcal disease[90]. Regulatory cells have come to the forefront in the explanation as to why some children still see persistence of carriage despite colonisation events which would have provided them with a robust effector T cell response and in theory should allow for the clearance of the bacteria. Regulatory T cells are very important for preventing an excessive pro inflammatory response. They prevent self-tissue damage, but their presence also contributes to the persistence of carriage[90]. It has been suggested that specific pneumococcal components could be responsible for the induction of regulatory T cells in the nasopharynx and studies have shown that stimulation of adenoidal cells with pneumococcal whole cell antigen does show a significant increase in the numbers of regulatory T cells observed and in those samples which are culture positive for pneumococcus the increase is significantly higher[90]. This suggests the local colonisation of the nasopharynx with the bacteria causes antigen specific induction of Treg cells.

Understanding the regulation of naturally-acquired mucosal immunity should help inform the design of novel vaccination strategies against pneumococcal colonization and/or

infection. Currently available vaccines target specific capsular polysaccharide and their efficacy is limited as only a certain number of serotypes are included in the vaccine[47]. Polysaccharide vaccines are also limited by the fact they induce a T cell independent antibody response which is ineffective in children under 2 years of age[4, 12]. This issue was overcome by the formation of polysaccharide conjugate vaccines in which the polysaccharide is conjugated to a protein, however these vaccines are also limited by the serotypes included and are too expensive for routine use in developing countries. These reasons clearly explain why there is need to develop a suitable protein vaccine which would be effective against all serotypes and this is a key priority in pneumococcal vaccine research. There are several pneumococcal proteins that have been identified as potential targets for inclusion in a pneumococcal protein vaccine and numerous studies have shown their protective efficacy in animal models[303-305].

Pneumolysin is a protein which is highly conserved amongst *S. pneumoniae* isolates and is able to destroy cells by forming pores in cell membranes that contain cholesterol[47]. Studies in experimental mice models have shown that pneumolysin can induce protective immunity against pneumococcus[200, 202, 203]. This PhD project has investigated the role of pneumolysin and capsular polysaccharide in the induction of regulatory T cells in human tonsillar tissue and blood samples.

During the last decade, research into Foxp3+ Treg cells has grown, showing that these cells play an important role in regulation during infections. However, if a cell response is skewed towards regulatory cells, persistence of infection can be seen[306]. Foxp3+ Treg cells have a close and finely balanced relationship with Th17 cells, which are of an effector phenotype and act to clear infection. This relationship is tightly controlled, with an imbalance playing a role in several diseases[88, 307, 308]. It is also interesting to note that the differentiation of both cells types involves a key role for TGF-β[309].

Recent studies have reported that the numbers of Foxp3+ Treg cells in adenotonsillar tissue varies depending on the presence or absence of pneumococci, which indicates that carriage has an effect on this cell type[90, 121]. In people who have asthma, a reduced number of Treg cells are observed, with those that are present having a reduced functional capacity[310, 311]. Treg cells act to regulate inflammation and as such their presence could be an effective therapy for fighting diseases such as asthma. With this in mind studies have been undertaken which have attempted to harness the regulatory capacity of Treg cells in order to control asthma[312]. Mouse models using live or killed pneumococcus or pneumococcal vaccines have been used and have been shown to reduce the hallmarks of allergic airways disease suggesting certain pneumococcal components may be important for inducing regulatory T cells[313-315]. A combination of Type 3 polysaccharide and a pneumolysin toxoid which were co administered in a mouse model at various intervals showed a significant decrease in the hallmark AAD characteristics and were able to induce Treg cells in significantly higher levels compared to controls and compared to when each was administered separately[316]. Using this information, I designed my own investigation looking at the effects of pneumococcal polysaccharide and a toxoid of pneumolysin (in this case, W433F). I used the polysaccharide and pneumolysin toxoid together or separately. Interestingly, in my studies I did not see an increased activation of Foxp3+ Treg cells when polysaccharide plus pneumolysin toxoid were used to stimulate cells together. However, each individual component used showed a significant increase in Foxp3+ Treg cell numbers compared to an unstimulated control.

Chapter 4 investigated the relationship between Foxp3+ Treg cells and pneumococcus by stimulated tonsillar MNC with pneumococcal CCS, purified capsular polysaccharide and pneumolysin toxoid, W433F. It was observed that pneumococcal capsular polysaccharide and pneumolysin are able to stimulate an increase in Foxp3+ Treg in adenotonsillar tissue

(Figure 4.4.5). There is limited data about the role of regulatory T cells and their relationship with pneumococcus during colonisation of the nasopharynx. Data relating to human samples is particularly limited. A previous study showed the pneumococcal whole cell antigen (WCA) was able to induce Foxp3+ Treg cell proliferation in tonsillar MNCs which suggests there are pneumococcal protein/s which could have a role to play in the induction of these cells.[90] Currently, it remains unclear which pneumococcal proteins may have a role in the activation or induction of regulatory T cells in in human nasopharynx-associated tissue (NALT). The identification of such protein/s would be useful information to further our understanding on the role of Treg in pneumococcal carriage and on novel vaccination strategies against pneumococcal disease.

Pneumococcal vaccine research has become more focussed on identifying pneumococcal proteins which could be included in a vaccine that would then be effective against all known serotypes[240, 264, 265]. Pneumococcal proteins including pneumolysin and choline binding protein A have been studied to see if they could be used in a vaccine[198].

In this study we investigated the importance of pneumolysin and capsular polysaccharide in the activation of Foxp3+ Treg cells. I initially stimulated tonsillar MNC with pneumococcal CCS with and without pneumolysin and capsule to determine their importance in Foxp3+ Treg cell activation. Stimulation using D39 WT strain of pneumococcal CCS showed significantly higher activation of Foxp3+ Treg cells (Figure 4.4.4) compared to both mutant strains used. The first mutant strain was pneumolysin (Ply) deficient (Ply -/-) and the second was capsule deficient (Cap -/-). These initial results suggested that both pneumolysin and capsular polysaccharide could be important for the activation of Foxp3+ Treg cells.

After the initial findings using pneumococcal CCS I wanted to look at whether purified capsular polysaccharide and the toxoid of pneumolysin, W433F, could activate Foxp3+ Treg cells compared to a negative control. My results show that W433F and purified capsular

polysaccharide are able to activate pre-existing Foxp3+ Treg cells to significantly higher levels compared to an unstimulated control. Two different types of capsular polysaccharide were used, T3P and 6B and both were shown to activate Foxp3+ Treg cells compared to the negative control. Capsular polysaccharide is currently used in pneumococcal vaccines and we know it is effective at preventing pneumococcal disease. In my results, pneumolysin has been shown to be effective at activating Foxp3+ Treg cells. As it is expressed by most if not all clinical isolates of pneumococcus, the inclusion of pneumolysin in a vaccine would potentially confer protection against most serotypes whilst remaining relatively cheap to produce.

The pre-existing Foxp3+ Treg cells detected in tonsillar tissue are likely to be those which are primed due to previous pneumococcal colonisation events in the nasopharynx. The ability of both W433F and both capsular polysaccharides used to activate these cells suggests that both the polysaccharide capsule and pneumolysin can activate these cells and therefore play a role in the carriage of pneumococcus in the nasopharynx.

Tr1 cells have been shown to have a role to play in the regulation of various mucosal sites including the gut[317]. These cells are distinct from Foxp3+ Treg cells, lacking expression of the key transcription factors, Foxp3, and are suppressive through the production of IL10 and TGF-β[106]. Tr1 cells are differentiated in the periphery and are activated in an antigen specific manner where they are able to regulate both naïve and memory T cell responses[105, 106]. Tr1 cells require activation through their T cell receptor to exert their suppressive effects and this happens in an antigen specific manner[105, 277]. Once activated however, they exert their effects in a non-specific way[277].

Mouse models have suggested that cellular therapy with Tr1 cells is feasible[318]. Translating the success of mouse models into a successful human therapy has its challenges but evidence is increasing that Tr1 cells could be a feasible immunomodulatory therapy for a

variety of human diseases[277, 318]. Tr1 cells have been shown to be able to suppress antigen specific CD4+ T cell proliferation. It was also shown that Tr1 cell clones transferred into SCID mice could prevent experimentally induced colitis[105, 155]. IL10 is able to act to directly inhibit cytokines that would induce antigen specific CD4+ T cells as well as being able to inhibit antigen presenting cells, both of which reduce inflammation[319].

A recent a phase I/IIa clinical trial has been performed in which Tr1 cells have been assessed for their impact on Crohn's Disease[320]. Ovalbumin specific Tr1 cells have been shown to reduce in vivo inflammatory responses in animal models to prevent and control colitis[105, 321]. The phase I/IIa clinical trial was performed to assess the efficacy and safety of ovalbumin Tr1 cell injections in Crohn's disease patients and showed that the Crohn's disease activity index of patents injected was significantly reduced[320].

Clearly Tr1 cell research has become much more intensive recently and understanding how this cell type is involved in numerous diseases and auto immune disorders is important to be able to successfully create effective therapies. The role of Tr1 cells in pneumococcal disease hasn't been investigated but the fact Foxp3+ Treg cells have such an important role to play could mean other regulatory T cells are also involved. Tr1 cells could have a role to play in the carriage of pneumococcus and the persistence of infection.

Chapter 5 investigated the relationship between pneumococcus and Tr1 cells in human tonsillar tissue. We investigated the importance of pneumolysin and capsular polysaccharide in the activation and induction of Tr1 cells. To determine whether capsular polysaccharide and pneumolysin had a role to play in Tr1 cell activation, I initially stimulated human tonsillar MNC with pneumococcal CCS. Stimulation using D39 WT strain of pneumococcal CCS showed significantly higher activation of Tr1 cells (Figure 5.4.3) compared to both mutant strains used. The first mutant strain was pneumolysin (Ply) deficient (Ply -/-) and the second was capsule deficient (Cap -/-). These initial results

suggested that both pneumolysin and capsular polysaccharide could be important for the activation of Tr1 cells.

After the initial findings using pneumococcal CCS I wanted to look at whether purified capsular polysaccharide and the toxoid of pneumolysin, W433F, could activate Tr1 cells compared to a negative control. Figure 5.4.4 shows that the pneumolysin toxoid W433F and both capsular polysaccharides used (T3P and 6B) were able to elicit a significant increase in Tr1 cell numbers compared to an unstimulated negative control. The combination of W433F and T3P or W433F and 6B didn't show any more significant activation than each component alone which is interesting as it seems to suggest they do the same thing without having an additive effect. It had previously been shown in a mouse model that the combination of T3P and Ply did confer an increased protective effect when compared to either the T3P or Ply alone[127].

This research looking at Tr1 cells responses to pneumococcal components is novel. There is already lots of research looking at Foxp3+ Treg cells responses but it is interesting to see that another category of regulatory T cells are reacting in much the same way. The stimulation using pneumolysin and capsular polysaccharide increased the numbers of Tr1 cells significantly which indicate their importance for Tr1 cell activation. The presence of pre-existing Tr1 cells could be due to previous colonisation events by the pneumococcus in the nasopharynx. This would explain why stimulation saw the numbers of these cells increasing as fresh stimulation would activate cells that had previously encountered the stimuli.

To determine if Tr1 cells could be induced, we depleted samples of CD45RO+ cells. As with the activation experiments, initially cells were stimulated using pneumococcal CCS (Figure 5.4.5) to see whether any differences were observed in induction with and without pneumolysin and capsular polysaccharide. We did in fact once again see that when Ply-/-

and Cap-/- CCS were used, the induction of Tr1 cells was significantly less than that when cells were stimulated using D39 WT pneumococcal CCS. This once again suggested pneumolysin and capsular polysaccharide are important players in Tr1 cell responses. So, I then moved on to stimulation with purified pneumolysin toxoid (W433F) and purified capsular polysaccharide (T3P and 6B). (Figure 5.4.6) W433F stimulation alone showed significantly more Tr1 cells induction compared to the negative control. T3P and 6B stimulation alone also showed significantly more Tr1 cells induction compared to the negative control. And again, the combination of W433F with either of the two polysaccharides did not have an increased induction and was no different from to either components alone.

These results mirror those seen using pneumolysin and capsular polysaccharide for the activation of Foxp3+ Treg cells suggesting that several categories of regulatory cells are affected by pneumococcus. In this study, 2 different types of purified capsular polysaccharide were used to stimulate cells, T3P and 6B, and both were able to activate pre-existing Tr1 cells and induce Tr1 cells from naïve T cells. Pneumolysin was also able to both activate and induce Tr1 cells to much the same level as the capsular polysaccharide used. These results suggest Tr1 cells could have a role to play in the carriage of pneumococcus.

The pre-existing Foxp3+ Treg and Tr1 cells detected in tonsillar tissue are likely to be those which are primed due to previous pneumococcal colonisation events in the nasopharynx. The ability of both W433F and both capsular polysaccharides used to activate these cells suggests that both the polysaccharide capsule and pneumolysin are able to activate these cells and therefore play a role in the carriage of pneumococcus in the nasopharynx.

The ability of pneumolysin to activating pre-existing Foxp3+ Treg and Tr1 cells suggests that this pneumococcal protein does specifically influence the immune response generated in response to pneumococcal exposure. Understanding the precise mechanism of these

interactions and its effects on other immune cells is key to further research, which aims to include this protein in any future vaccines.

In addition to this, my research has focussed on the interactions between pneumococcus and regulatory cells in the nasopharynx. Given that the nasopharynx is the site of pneumococcal colonisation, that prolonged carriage of the bacteria leads to spread throughout the community and that disease cannot occur without colonisation, the nasopharynx could be a potential target for a future vaccine that directly works at the mucosal site rather than through the blood. Again, it is important to understand the balances and intricacies of how any potential vaccine would work and how it would affect the hosts' natural response, but this is certainly an interesting and potentially rewarding area of research which could have very fruitful results in the future.

The overall findings in this study support the hypothesis that both pneumococcal polysaccharide and pneumolysin have an important role in the activation of Foxp3+ Treg and Tr1 cells in the nasopharynx. They suggest that in the mucosal tissue of the nasopharynx pneumolysin and capsular polysaccharide can promote regulatory T cells which contribute to the persistence of carriage and the delay in pneumococcal clearance.

TGF-β is thought to be important in the conversion of naïve CD4+ T cells into Foxp3+ Treg cells which can then proliferate and lead to suppression of the immune system[284, 285]. A study by Liao et al, showed that SPY1, a live attenuated vaccine against pneumococcus, is able to induce protective Treg cells[286] which are vital for the maintenance of homeostasis of the immune system and prevent infection associated inflammation[287]. In addition to this they showed that the addition of a peptide, P17[286, 288], which inhibits TGF-β and therefore reduces the numbers of Foxp3+ Treg cells, impairs the effectiveness of SPY1 which suggests the ability of SPY1 to activate TGF-β and activate Treg cells is important for producing protective Treg immune responses in the nasopharynx[287]. This suggests TGF-β has an

important role to play in the activation of Foxp3+ Treg cells and the carriage of pneumococcus in the nasopharynx. This protective role of regulatory T cells has also been shown in a mouse study by Neill et al[275]. This study showed that regulatory T cells have an important role to play in the prevention of immune mediated damage in the lungs of infected mice and that TGF-β has an important role to play in this[275]. They used 2 mice models which react very differently to pneumococcal infection[289] and infected the lungs with pneumococcus. CBA/Ca mice which have fewer T regulatory cells and therefore less TGF-β showed insufficient immunosuppression and the bacteria was able to disseminate into the bloodstream before they could be cleared. In contrast, in BALB/c mice, a controlled and regulated pro-inflammatory environment was created quickly after infection and bacteria were cleared. The presence of Treg cells in this environment acted to limit pro inflammatory responses to prevent tissue damage and maintain the integrity of the lungs and prevent bacteria getting into the bloodstream[275].

It has been suggested that TGF-β has a role to play in the activation of Foxp3+ Treg cells and in this study, we investigated the possible role of integrin, avb8 on the activation of TGF-β and the effect of this on the activation of Foxp3+ Treg cells.

Initially, tonsillar MNC were stimulated with WT pneumococcal CCS before the supernatant was analysed for the presence of TGF-β and cells were stained by intracellular cytokine staining to detect the presence of Foxp3+ Treg cells. My results show that when freshly isolated tonsillar MNC are stimulated using WT pneumococcal CCS the concentration of TGF-β is increased compared to a negative control with no stimulation (Figure 6.4.1). FACS analysis of the cells showed that the percentage of Foxp3+ Treg cells increased when cells were stimulated with WT pneumococcal CCS compared to an unstimulated sample, which correlates with the increase in TGF-β concentration seen.

Tonsillar MNC stimulated with WT pneumococcal CCS after pre-incubation with anti-avb8 and anti-TGF beta both showed decreased levels of TGF-β when compared to cells stimulated with WT pneumococcal CCS alone. FACS analysis also revealed that the cells stimulated after pre-incubation with anti-TGF-β and anti-αvβ8 showed a reduction in the percentage of Foxp3+ Treg cells present compared to cells stimulated using WT pneumococcal CCS alone. These combined results seem to suggest a cascade of activation for Foxp3+ Treg cells, with αvβ8 being important for the activation of TGF-β which in turn is needed for the activation of Foxp3+ Treg cells.

Knowing the mechanism through which Foxp3+ Treg cells exert their suppressive effects would allow for a more informed therapeutic strategy against pneumococcal infection. The nasopharynx has long been established as the site of colonisation of pneumococcus with more colonisation events occurring in the early years of life[90] when infection can be more severe.

A protective role of regulatory cells in pneumococcal disease and infection has been shown in several studies[275, 287] as has already been mentioned. The fact that regulatory T cells are clearly playing such an important role in pneumococcal disease and carriage just highlights how important it is for us to understand the immunology of pneumococcal infection, both of effector and regulatory T cells. A clear and complete picture is needed to be able to create an effective therapeutic strategy against infection. Vaccination has proven to be an effective strategy against pneumococcus and the evolution of pneumococcal vaccines from polysaccharide vaccines to conjugate vaccines has shown effective at reducing the instances of colonisation an invasive disease[29, 178, 187 188, 189]. Current pneumococcal vaccines can be administered by injection or mucosally but are mainly delivered via injection[287]. A mucosal vaccine which is administered directly into the nose could prove to be an effective way of stimulating both a systemic immune response and a mucosal immune responses

whereas an injected vaccine only stimulates the systemic immune system and therefore doesn't offer an effective way affecting the carriage and clearance of the bacteria from the nasopharynx [287].

The results here all underlines the importance of knowing what regulatory immune responses are elicited by pneumococcus and how they are activated. It is clear they are playing a role during pneumococcal colonisation and disease and understanding these cell responses could be important for the future therapeutic strategy against pneumococcal colonisation and disease.

7.2 Conclusion

Results presented in this study show that there are significant numbers of both Foxp3 regulatory and Tr1 cells in human tonsillar tissue which could have an important role in mediating the carriage of pneumococcus and as such be an important target for vaccines to prevent carriage. It was also shown that the numbers of Foxp3+ Treg and Tr1cells are significantly higher in children tonsil samples compared to adult tonsil samples. Children are known to have multiple colonisation event throughout their early years, a phenomenon that decreases with age. This study suggests a relationship between the age of the patients and the number of regulatory T cells in the nasopharynx where one influences the other. The decrease in colonisation seen with age could be either due to the reduced number of regulatory cells in the nasopharynx the numbers of regulatory cells in the nasopharynx are reduced due to a reduce number of colonisation events.

The stimulation of human tonsillar MNC with pneumococcus showed a significant increase in both Foxp3 regulatory and Tr1 cells, which suggests antigen specific Treg cells may be present in the nasopharynx, presumably because of previous colonisation events. Results presented in this study show that pneumolysin and capsular polysaccharide may contribute to the activation of Foxp3+ Treg cells and to the activation and induction of Tr1 cells in

human NALT and have a role to play in the modulation of carriage of the bacteria It has also been shown that TGF-β has a role to play in the activation of Foxp3+ Treg cells and that the activation of the TGF-β is affected by the presence of the integrin, αvβ8. Adenotonsillar MNC stimulated by WT pneumococcal CCS in the presence of anti- TGF-β and anti- αvβ8 show a significantly lower concentration of TGF-β and a significantly lower percentage of Foxp3+ Treg cells.

In conclusion, these findings add information to our understanding of how pneumolysin and capsular polysaccharide mediate the activation of regulatory T cells, information that could be used in the development of future vaccines against pneumococcal disease. Understanding the complete immune response against pneumococcus and how the immune response reacts to stimulation using pneumococcal antigens could have implications in future vaccination strategies against pneumococcus. A vaccine that is directly applied to the nasopharynx could be effective at regulating carriage of the bacteria and thus preventing its spread through the community.

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Appendices

Appendix-I

Preparation of different media and buffers

Skim milk, Tryptone, Glucose, Glycerol Transport Medium (STGG)

STGG transport medium was prepared using the following method.

1. 40 ml 10% milk was prepared by adding 4 grams of skimmed milk powder (Oxoid, UK) into 40 ml of distilled water in a sterile 100 ml glass bottle.
2. The bottle was autoclaved at 121°C for 5 minutes with the cap loosened.
3. Upon completion of autoclaving, the cap was tightened, and the bottle taken into a bio-safety cabinet.
4. In another sterile 500 ml glass bottle, 6 ml of Tryptone soya broth (Oxoid, UK), 2.2 ml of 45% D-Glucose solution (Sigma Aldrich, UK) and 20 ml of Glycerol (Sigma Aldrich, UK) were added with 131.8 ml of distilled water to make the final volume 160 ml.
5. The bottle was shaken gently to dissolve all ingredients and the bottle was then autoclaved at 121°C for 10 minutes with the cap loosened.
6. After that, the cap was tightened, and the bottle was taken into a bio-safety cabinet.
7. Then, the previously prepared sterile 10% skimmed milk (40 ml) was added into the 500 ml bottle to make 200 ml of 2% skimmed milk medium.
8. The milk was mixed thoroughly with the other contents by gentle shaking of the bottle.
9. Aliquots of 1.0 ml media were prepared into 1.5 ml screw-capped vials.
10. The tubes were then kept in a refrigerator (2-8°C) until used for nasal swab inoculation.
11. Quality control test for sterility of the STGG medium was performed periodically by plating a full loop of a homogenized vial from each lot onto a blood agar plate, then incubating the plate at 37°C overnight. If growth of any organisms was suspected, the entire batch was disposed of.

Todd-Hewitt-Yeast Broth (THYB)

Todd-Hewitt Broth with 0.5% yeast extract was prepared using the following procedure.

1. 36.4 grams of Todd-Hewitt Broth (Oxoid, Basingstoke, UK) and 5 grams of yeast extract were added to a sterile 1 litre glass bottle.
2. 1000 ml of distilled water was added to the bottle and mixed thoroughly, until all components were fully dissolved.
3. The bottle was autoclaved at 115°C for 10 minutes with the cap loosened.
4. After autoclaving, the bottle cap was closed tightly and then kept in the cold room until used for growing bacteria.

Laemmli Reducing Buffer

10.0 ml Laemmli reducing Buffer for western Blot sample dilution was prepared using the following recipe ingredients:

0.5 M Tris pH 6.8	1.25 ml
10% (w/v) SDS	2.0 ml
0.5% Bromphenol Blue	0.20 ml
Glycerol	2.50 ml
Deionized H2O	3.55 ml
β-mercaptoethanol	0.50 ml (50 µl for each 0.95 ml)

10x Tris Buffered Saline (TBS) pH 7.4

1 Litre 10x TBS was prepared in a sterile 1 litre glass bottle, using the following ingredients:

Tris	30 gm
NaCl	80 gm
KCl	2 gm
Deionized H2O	900 ml

The pH was adjusted to 7.4 by adding concentrated HCl drop by drop, with gentle shaking of the bottle and measurement with a pH meter. When the pH was adjusted to the correct level, the bottle was topped-up to 1 litre by adding deionized water.

Western Blot Washing Buffer (TBS-T)

1 litre of Western Blot washing buffer (1xTBS with 0.05% Tween20) was prepared in a sterile glass bottle, using the following method:

10x TBS	100 ml
Deionized H2O	900 ml
Tween-20	0.5 ml

Western Blot Blocking Buffer (5% skimmed milk in TBS-T)

500 ml of Western Blot blocking buffer (5% skimmed milk in TBS-T) was prepared in a sterile glass bottle, with a magnetic stirrer bead inside, using following recipe-

Skimmed milk	25 gram
10x TBS	50 ml
Deionized H2O	450 ml
Tween-20	0.25 ml

The bottle was kept on a magnetic stirrer which allowed the milk to dissolve effectively.

10x Phosphate Buffered Saline (PBS) pH 7.4

1 litre of 10x PBS was prepared in a sterile 1 litre glass bottle, using the following ingredients:

Na2HPO4	14.4 gm
KH2PO4	2.4 gm
NaCl	80 gm
KCl	2 gm
Deionized H2O	900 ml

The pH was then adjusted to 7.4 by adding concentrated HCl drop by drop, with gentle shaking of the bottle and measurement with a pH meter. When the pH had been adjusted to the correct level, the bottle was topped up to 1 litre with deionised water.

ELISA/ELISpot Washing Buffer (PBS-T)

1 litre of ELISA/ELISpot washing buffer (1xPBS with 0.05% Tween20) was prepared in a sterile glass bottle, using following recipe-

10x PBS	100 ml
Deionized H2O	900 ml
Tween-20	0.5 ml

P-Nitrophenyl Phosphate (PNPP) Substrate for ELISA

1 litre of p-nitrophenyl phosphate (PNPP) substrate (1M diethanolamine) buffer (pH 9.8) was prepared in a sterile glass bottle, using following recipe-

Diethanolamine	97 ml
Deionized H_2O	800 ml
MgCl2	100mg

The bottle was then placed on a magnetic stirrer with a magnetic flea inside to allow for the contents to thoroughly mix. Whilst stirring, the pH was measured and adjusted to 9.8 by adding 10M hydrochloric acid. Once the pH was adjusted to the correct level, the buffer was transferred to a measuring cylinder and topped up to 1 litre using distilled water.
The buffer was then transferred back to the bottle and stored at 4˚C. Finally, the substrate was prepared by dissolving a p-nitrophenyl phosphate disodium salt (5mg) tablet into 5 ml of substrate buffer to give a PNPP concentration of 1 mg/ml.

Appendix II

List of antibodies/chemicals and reagents/Consumables

Antibodies/kits	Manufacturer	Catalogue no
Anti-rabbit IgG-HRP (produced in donkey)	Santa Cruz Biotech, Germany	SC2077
CD45RO microbeads (mouse anti-human IgG2a)	Miltenyi Biotech, Germany	130-092-355
Human CD-4 PE (clone: RPAT4)	BD Biosciences, UK	555347
Human CD-4 PE-Cy7 (clone: SK3)	BD Biosciences, UK	557852
Human CD49b AlexaFluor647 (clone: 12F1)	BD Biosciences, UK	564118
Human Foxp3 AlexaFluor647 (clone: 259D/C7)	BD Biosciences, UK	560045
Human IL-10 APC (clone: JES3-19F1)	BD Biosciences, UK	554707
TGF-β-1,2,3 Monoclonal Antibody (1D11.16.8) functional grade	eBioscience, UK	16-9243-85
Human LAG-3 PE	R&D Systems	FAB2319P

Chemicals/Reagents	Manufacturer	Catalogue Number
Agar	Sigma Aldrich, UK	A1296
Agarose	Sigma Aldrich, UK	A9539
Amphotericin B	Sigma Aldrich, UK	A2942
Blood Agar Base	Oxoid Ltd, UK	CM0055B
Bovine Serum Albumin (BSA)	Sigma Aldrich, UK	A8327
Bradford Protein Dye Reagent	Sigma Aldrich, UK	B6916
Brefeldin A	eBioscience, UK	00-4506-51
Phosphate Buffered Saline (PBS) (tablet)	Sigma Aldrich, UK	P4417
Ficoll-Plaque Premium	GE Healthcare Life Sciences, UK	17-5442-03
Foetal Bovine Serum (FBS)	Sigma Aldrich, UK	F7524
Foxp3/Transcription factor staining buffer set	eBioscience, UK	00-5523-00
Gentamycin	Sigma Aldrich, UK	G1272
Glucose	Sigma Aldrich, UK	G8769
Hanks Balanced Salt Solution (HBSS)	Sigma Aldrich, UK	H9269
Horse Blood (Defibrinated)	Oxoid Ltd, UK	SR0050C
Horseradish Peroxidase Avidin D	Vector Lab. Inc., USA	A-2004

Hydrogen Peroxide	Sigma Aldrich, UK	H1009
Intracellular fixation buffer	eBioscience, UK	00-8222-49
Immun-Star WesternC Chemiluminescent Kit	Bio-Rad, UK	170-5070
L-glutamine	Sigma Aldrich, UK	G7513
NN Dimethylformamide (DMF)	Sigma Aldrich, UK	D4551
Optochin disk	Oxoid Ltd, UK	DD0001B
Penicillin/Streptomycin	Sigma Aldrich, UK	P0781
Permeabilisation buffer (10x)	eBioscience, UK	00-8333
Quick-load DNA Ladder (1KB)	New England Biolabs, USA	N3232L
RPMI 1640	Sigma Aldrich, UK	R5886
Skimmed Milk Powder	Oxoid Ltd, UK	LP0031B
Streptactin-HRP	Bio-RAD, UK	61-0381
Tryptone Soya Broth	Oxoid Ltd, UK	B00369E
Yeast Extract	Sigma Aldrich, UK	Y1625

Consumables	**Manufacturer**	**Catalogue Number**
BD Falcon cell strainer (70µm, nylon)	BD Biosciences, UK	352350
Columbia blood agar plate	Oxoid Ltd, UK	OXPB 0199A
Dryswab™ in peel pouch	Medical Wire & Equipment, UK	MW113
ELISA plates (96 well, flat, high protein binding EIA/RIA plate)	Corning, USA via Appleton Woods, UK	CC679
MACS cell separation LD columns	Miltenyi Biotech Ltd, Germany	130-042-901
Mini Protean precast TGX gel (12%)	Bio-Rad, UK	456-1044
Oracol saliva collection system	Malvern Medical Dev. Ltd, UK	510
Transblot turbo mini transfer pack	Bio-Rad, UK	170-4156
Vivaspin 15R hydrosart (MWCO: 5000)	Sartorius Stedim, Germany	VS15RH11